From Department of Laboratory Medicine
Clinical Research Centre - Experimental Cancer Medicine
Karolinska Institutet, Stockholm, Sweden

MICROARRAY GENE EXPRESSION IN IMMUNOLOGICAL CONDITIONS

Heevy Abdulkareem Musa
Al-Chaqmaqchi

Stockholm 2013

Published by Karolinska Institutet. Printed by Eprint AB

ISBN 978-91-7549-146-2

To my family

ABSTRACT

The use of microarray techniques for gene expression analysis provides a global view of the transcriptional activity in a biological sample. In this thesis two conditions accompanied by disturbance in the immune system are addressed following the changes in gene expression. The first condition is hematopoietic stem cell transplantation (HSCT) and the second condition is chronic kidney disease (CKD). A mouse model was used in studies related to HSCT, while human blood samples were used in the CKD study.

In the first project, gene expression profiling in target and non-target organs after HSCT and during early development of acute graft versus host disease (GVHD) was investigated. The results show that both chemotherapy and allogeneicity induce expression of inflammatory genes in target tissues. Furthermore, gene expression in the kidney was similar to that observed in the liver, which is a target for developing GVHD. In conclusion, the kidney could be a possible target organ for GVHD.

In the second project, the role of programmed cell death ligand-1 (PD-L1/CD274) in the development of graft versus host disease was studied. The expression of PD-L1/CD274 was evaluated by PCR, western blot and immunohistochemistry at different time intervals after the development of acute GVHD. A significant up-regulation of PD-L1/CD274 expression was observed in muscle and kidney at days 5 and 7 post allogeneic transplantation. The increase of CD274 expression was paralleled by high serum levels of IFN-γ and TNF-α at corresponding time points. In conclusion, our results confirm that PD-L1/CD274 expression is different after allogeneic and syngeneic transplantation, and that it is more expressed in non-target organs in the early stages of acute GVHD. This further indicates that the higher expression of PD-L1/CD274 in the muscle might have a protective role during acute GVHD.

In the third project, changes in gene expression after two different conditioning regimens used in preparation for HSCT, either single dose or fractionated total body irradiation (TBI), were studied. In the lung, genes belonging to inflammatory and immune responses were intensely under-expressed upon conditioning with single-dose, but slightly increased by treatment with fractionated TBI. These genes remained unchanged in the liver and muscle upon conditioning with either of these regimens. Both the patterns and magnitudes of these effects were changed after 12 and 72 hours. Thus, pathological manifestations observed in the lung after conditioning with single-dose, but not fractionated, TBI can be associated with extensive down regulation of immune and inflammatory gene expression in this organ.

In the fourth project, gene expression profile for monocytes from peripheral blood of chronic kidney disease (CKD) patients was performed. The results demonstrated differences from the gene expression profile of healthy persons. Pathways involved in the inflammatory response were highly expressed in CKD patients, and the Wnt/β-catenin signaling pathway was the most significant pathway expressed in the patient group. Since this pathway has been attributed to a variety of inflammatory manifestations, the current findings may contribute to dysfunctional monocytes in CKD patients. Strategies for interfering with this pathway may improve host immunity and prevent cardiovascular complications in CKD patients.

Contents

LIST OF PUBLICATIONS

I. Behnam Sadeghi, **Heevy Al-Chaqmaqchi**, Sulaiman Al-Hashmi, David Brodin, Zuzana Hassan, Manuchehr Abedi-Valugerdi, Ali Moshfegh, Moustapha Hassan. Early phase GVHD gene expression profile in target versus non-target tissues: kidney, a possible target? Bone Marrow Transplant. 2013 Feb; 48(2): 284-93.

II. **Heevy Al-Chaqmaqchi,** Behnam Sadeghi, Manuchehr Abedi-Valugerdi, Sulaiman Al-Hashmi, Mona Fares, Raoul Kuiper, Joachim Lundahl, Moustapha Hassan*, Ali Moshfegh*. The role of programmed cell death ligand-1 (PD-L1/CD274) in the development of graft versus host disease. PloS one, 2013, 8.4: e60367.

III. **Heevy Al-Chaqmaqchi**[a], Sulaiman Al-Hashmi[a], Behnam Sadeghi, Zuzana Potácová, Joachim Lundahl, Ali Moshfegh, Manuchehr Abedi-Valugerdi*, Moustapha Hassan*. Gene expression profiling in the liver, lung and muscle of mice conditioned with single-dose or fractionated total body irradiation. [Manuscript]

IV. **Heevy Abdulkareem Musa Al-Chaqmaqchi,** Ali Moshfegh, Elham Dadfar, Josefin Paulsson, Moustapha Hassan, Stefan H Jacobson, Joachim Lundahl. Activation of Wnt/β-catenin pathway in monocytes derived from chronic kidney disease patients. [Under review in PloS one]

[a] Equal contribution

* Shared senior authorship

LIST OF ABBREVIATIONS

Actb	β-actin
APC	Antigen presenting cell
BM	Bone marrow
BMT	Bone marrow transplantation
Bu	Busulfan
CD	Cluster of differentiation
cDNA	Complementary DNA
CKD	Chronic Kidney disease
Cy	Cyclophosphamide
DCs	Dendritic cells
FBS	Fetal bovine serum
GVHD	Graft versus host disease
GVL	Graft versus leukemia
Gy	Gray
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NK	Natural killer cells
PBS	Phosphate buffer saline
PD-L1	Programmed death ligand one
RIC	Reduced intensity regimen
TBI	Total body irradiation
TNF	Tumor necrosis factor

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system has developed over time to protect the body from environmental pathogens. It keeps the individual from infectious agents such as bacteria, viruses, fungi or parasites. It also works to control certain intrinsic pathologic agents, such as tumor cells. Moreover, different chemical and physical agents induce immune response.

The immune system consists of a highly organized network of organs, cells, and molecules. In healthy conditions, the immune system can distinguish foreign molecules from the body’s own and will attempt to destroy them. However, a strict control and regulation of the immune system is essential as both excessive and insufficient activation can be harmful, leading to autoimmunity and immune suppression respectively.

Traditionally, the immune system is described as being composed of two components: the innate and the adaptive system. When a pathogen invades the body it is faced by the first line of defense, which consists of physical and chemical barriers such as skin, mucosal surfaces and low gastric PH. If a pathogen passes these barriers, the cellular part of the innate immune system will be activated.[1] The innate immunity is unspecific and lacks memory.

The cellular part of innate immunity consists of different cells types that include neutrophils, natural killer cells (NK cells), monocytes, macrophages, dendritic cells (DCs), eosinophils, basophils, and mast cells. Together, these cells possess the ability to directly kill pathogens, produce compounds that enhance pathogen elimination and activate the adaptive branch of the immune system.

The activation of the innate immune system depends on the recognition of highly conserved microbial structures via antigen receptors specific for certain patterns on the microbes. An example of such receptors is the toll like receptors (TLRs).[2] Most pathogen invasions can be controlled by the innate immune cells, but sometimes extra response is needed. That is when the adaptive branch of the immune system becomes active.

The innate immune response is rapid and responds within hours, while the adaptive immune response takes more time (days to weeks) but is more powerful. It has a very high degree of specificity. Memory is the hallmark of adaptive immunity.[3] The adaptive immune system consists of lymphocytes and their products. It can be divided into cellular immunity mediated mainly by T cells expressing T cell receptors (TCRs), exerting cytotoxicity and producing cytokines, and humoral immunity mediated by B cells expressing B cell receptors (BCRs). The cells also have the ability to proliferate and clonally expand in response to an antigen in order to keep up with microbial replication. Lymphocytes are considered naïve before they meet certain cognate antigens, but when activated they can expand clonally and differentiate into many subtypes. After infection has been ended, most of the expanded lymphocyte populations die and balance is restored. Furthermore, some of the cells will be differentiated into memory cells that persist for many years and can respond very quickly upon re-infection by the same pathogen.

1.1.1 T cells

T-lymphocytes play an essential role in adaptive immunity. Precursors for T-lymphocytes are present and start developing in the bone marrow, after which they undergo developmental changes in the thymus. They are therefore called T-Lymphocytes (Thymus dependent). T cells have a receptor called T cell receptor (TCR) which is part of the T cell receptor complex. This complex is responsible for identification of the processed antigens presented by antigen presenting cells in the context of major histocompatibility complex (MHC) molecules. The TCR complex is formed by the TCR, CD3, and CD4 or CD8. Generally T cells are divided into CD4+ or CD8+ cells.

CD4+ T cells recognize antigens presented to them in the context of MHC class II molecules. They have the ability to produce different types of cytokines, which helps other cells to differentiate and mature.[4] After activation of CD4+ T cells, they can differentiate to different subsets such as Th1, Th2, Th17 and/or T regulatory cells, depending on the local environment, which are capable of inducing different kinds of immune response.[5-7]

The CD4+ T helper 1 cells (Th1) predominantly produce interferon gamma (IFN-γ), interleukin 2 (IL-2) and tumor necrosis factor alpha (TNF-α). These cells mediate cellular immunity through enhancement of microbiocidal activity by monocytes and macrophages and by promoting the differentiation of cytotoxic lymphocytes, as well as regulating certain B cells responses. The CD4+ T helper 2 cells (Th2) produce cytokines IL-4, IL-5, and IL-13, and by promoting mainly B cells to develop into antibody producing cytokines.[7]

The CD8+ cells (also called cytotoxic T cells) recognize antigens in the context of MHC class I molecules or altered self-cells, e.g. virus infected cells. When CD8+ cells activate, they produce large amounts of IFN-γ and TNF-α. They are very important in the killing of virus infected host cells, or intracellular microbes, as well as of tumor cells. They do not produce as much IL2 as CD4+ T cells, so they depend on CD4+ T cells to help them differentiate. The effects of IFN-γ produced by these T cells enhance the inflammatory response by activating macrophages and up regulating the expression of MHC classes I and II.[8]

1.1.2 Monocytes

Monocytes constitute 10% of peripheral blood leukocytes in humans and about 4% in mice. [9] Monocytes are members of the mononuclear phagocyte system.[10] They originate from CD34+ myeloid progenitor cells in the bone marrow and are released into the peripheral circulation, where they circulate for several days before entering tissues where they differentiate into macrophages or dendritic cells depending on the nature of environmental signals. For example, interferon-gamma switches monocyte differentiation from dendritic cells to macrophages.[11, 12] The process of migration of monocytes to peripheral tissues involves several adherence-detachment events mediated by selectins and integrins on monocytes and endothelial cells.[13]

Monocytes express CD14, which is a receptor for bacterial lipopolysaccharide (LPS). LPS binding to CD14 initiates transmembrane signaling and changes in cellular function.

Monocytes play a central role in immunity, as they link the inflammation and the innate response to adaptive immune response by their wide communications.[14] In addition they play an important role in development and homeostasis, via the removal of apoptotic cells and scavenging of toxic compounds. They are also implicated in many inflammatory diseases including atherosclerosis.[15, 16]

1.1.3 Antigen presenting cells and activation of T cells

Antigen-presenting cells (APCs) are specialized white blood cells that help to fight foreign substances that enter the body. First, the APC engulfs the antigen. Enzymes inside the APC break down the antigen into smaller particles. After that, the processed antigens are transported to the surface of the APC, and bound with an MHC class I or class II molecule. This complex forms epitopes that can be recognized and bound by a TCR in the immune system.[17]

The APCs are divided into two categories: professional and non-professional APCs. Professional APCs express MHC class II, while non-professional APCs express MHC class I.

There are three main types of professional antigen-presenting cells: macrophages, dendritic cells (DCs) and B-cells. These cells are able to engulf the antigen quickly during a process called phagocytosis.

Non-professional APCs include fibroblasts (skin cells), thymic epithelial cells, thyroid epithelial cells, glial cells (brain cells), pancreatic beta cells and vascular endothelial cells. While almost every cell in the body is technically an APC because they can present antigens to T-cells, the term is usually used to describe professional APCs.

When a T cell recognizes an antigen, the TCR binds to the peptide MHC complex in concert with either of the co-receptors CD8 or CD4, and this induces a signal in the T cell which is referred to as signal 1. The naïve T cell needs at least two signals from an APC to become activated and a third signal to differentiate into different effector subsets.

Signal 2 is the co-stimulatory signal that T cells receive from APCs to either promote or inhibit their survival and expansion. The most studied co-stimulatory molecules on the APCs are the B7.1 (CD80) and B7.2 (CD86) molecules that deliver signals via the CD28 molecule on the T cell.[18]

The synergistic activation of the naïve T cells with TCR engagement and co-stimulation via CD28 triggers the entry of the T cell into the G1 phase of the cell cycle, i.e. the initiation of cell division, and at the same time up regulation of the expression of IL-2 and the high affinity IL-2 receptor (CD25). IL-2 acts in an autocrine fashion to further clonally expand the T cell.[19]

When the T cells have become activated they express other co-stimulatory or inhibitory molecules that may modify the T cell response. ICOS and CTLA-4 are two such CD28-related proteins where the first stimulates and the second inhibits the T cell. Programmed death one (PD-1) is another CD28 analogue which binds to programmed death ligand 1 and 2 (PD-L1 and PD-L2) which also belong to the B7 family.[20] It inhibits T cell responses and mice lacking PD-1 develop autoimmunity. Another family of co-stimulatory molecules that up regulate after T-cell activation is the tumor necrosis factor superfamily (TNFSF).[8] Important receptor/ligand pairs included in this family are: CD137/CD137L, OX40/OX40L, CD30/CD30L, CD27/CD27L, and CD40/CD40L.

There are several mechanisms that inhibit the activation of T cells by self-antigens, the most important being the lack of co-stimulation. APCs that introduce self-antigens are usually not activated and do not express co-stimulatory molecules. Moreover, most tissue cells cannot express these molecules at all, and consequently the T cells cannot respond to the self-antigens. The T cells that recognize an antigen in the absence of co-stimulation enter a state of anergy, which is defined as a state of unresponsiveness with no proliferation or IL-2 secretion by T- cells. The anergic T cell cannot respond to the antigen even if it is introduced later in association with induced co-stimulation.[21] It is believed that this mechanism helps maintain peripheral self-tolerance. However, there are many exceptions to this role and self-reactive or auto reactive T cell clones are present in many autoimmune disorders. When activated T cells clonally expand they also initiate programs of differentiation that turn them into effector cells with specific functions. CD8 T cells become cytotoxic and CD4+ T cells differentiate into helper cells.

Signal 3 instructs the activated T cells to switch on a particular set of genes that further help differentiate the T cells. This signal is an environmental stimulus that is not very well defined in many cases; however, in most instances the cytokines produced by nearby tissue decide what direction T cells will take.

1.2 HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

The hematopoietic stem cell (HSC) is a pluripotent cell that has the capability to multiply to produce any type of blood cell in addition to its ability to renew itself and produce daughter HSCs. One of the features of HSCs is their capacity to return to the marrow space after intravenous infusion.[22] HSCs either develop into lymphatic progenitor cells from which T and B lymphocytes are derived, or into myeloid progenitor cells from which granulocytes (neutrophils, eosinophils, and basophils), monocytes, erythrocytes, and megakaryocytes are derived (Figure 1).

A single HSC can reconstitute the entire lympho-hematopoietic system in a syngeneic-transplanted animal after a dose of lethal total body irradiation.[23]

CD34 is a marker of HSC in humans, while in adult mouse bone marrow HSCs are detected in the CD34 low to negative fraction. CD34 low/- , c-Kit+, Sca-1 +, lineage markers negative (Lin-) are the usual markers for HSCs in mouse.[23, 24]

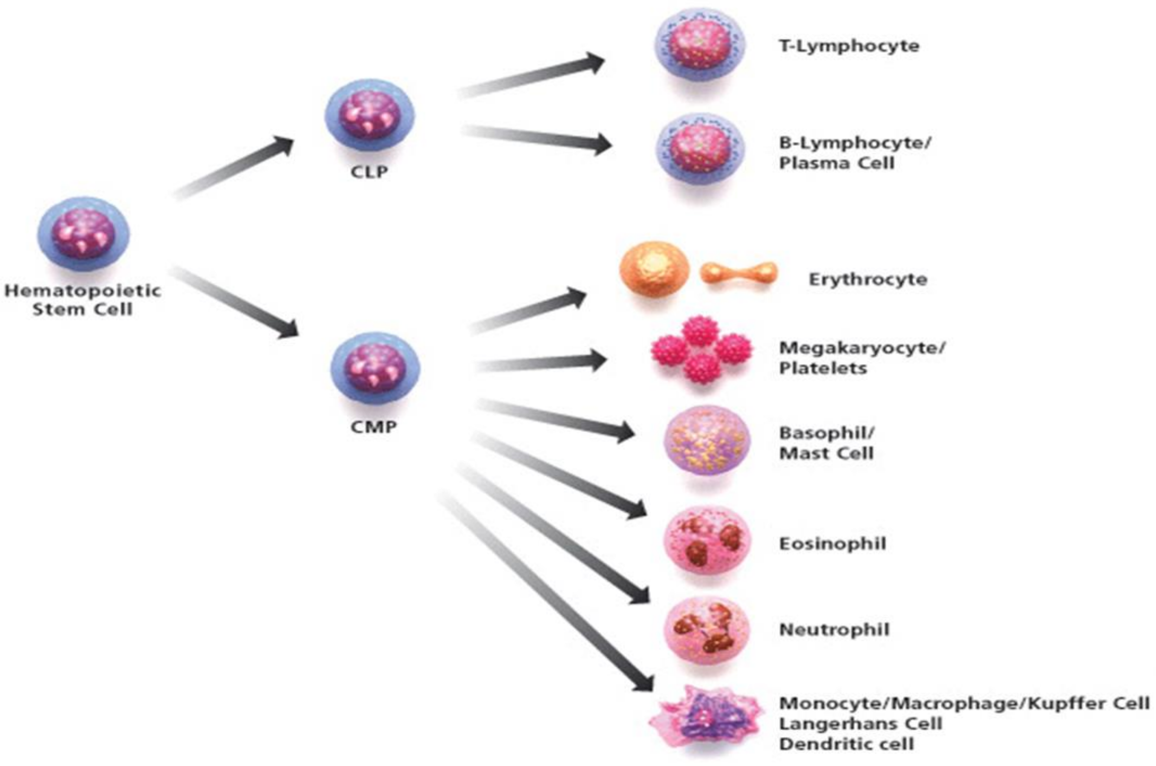

Figure 1. Hematopoietic stem cell. From: http://www.sigmaaldrich.com/life-science/stem-cell biology/hematopoietic-stem.html

HSCT can cure malignancy either by itself through immunological attack on cancer cells or by replacing the abnormal HSCs with normal ones.

HSCT consists of several steps including: elimination of recipient stem cells, eradication of tumor cells, suppression of the recipient immune system and finally transplantation of stem cells from a suitable donor. Several factors must be considered when determining HSCT for a patient. These factors include: health condition, disease stage, type of transplantation and type and dose of conditioning regimen.[25-27]

1.2.1 History of hematopoietic stem cell transplantation (HSCT)

In the last two decades, HSCT has become the curative treatment for many non-malignant diseases as for severe combined immune deficiency disease (SCID), thalassemia and sickle cell anemia, as well as malignant diseases and different metabolic disorders.[22, 28-32] The HSCT started with the observation that animals could escape death from a lethal dose of irradiation by protecting their spleen and/or their femur with a lead sheet.[33] Soon after, Lorenz and his colleagues found that protection could be also issued with an intravenous infusion of bone marrow.[34] The exact cause of the cure was unknown until later, when stem cells were identified. Early studies in humans were carried out by Donnall Thomas and colleagues in 1955.[35] Although the early studies were disappointing, they opened the way for wider studies. All these studies and experiments were carried out before we had knowledge of histocompatibility. They were based on inbred mice which do not require histocompatibility, which led to very high mortality if not a complete lack of success. After that in 1968 Storb, Epstein et al identified the canine major histocompatibility complex dog leukocyte antigen (DLA) and they showed that this determines the outcome of transplantation.[36] Furthermore, Epstein et al started to use cyclophosphamide in place of total body irradiation (TBI) for conditioning prior to transplantation.[37, 38]

1.2.2 Sources of HSC_s

Previously, bone marrow (BM) was the only source of HSCs for transplants, as there are ten times more of them in the BM than in the peripheral blood (PB). However, HSCs are still rare cells in the bone marrow. They represent 0.01% of all the cells in BM.[39] BM is not a solid organ, but rather diffuse and not easily accessible. During the last 20 years the majority of stem cell transplantation have been carried out using stem cells collected from peripheral blood after stimulation with granulocyte colony – stimulation factor (G-CSF).[40] Earlier engraftment of neutrophils and platelets has been reported in HSCT using PB stem cells compared to other stem cell sources.[41, 42] However, the use of PB stem cells showed an increased risk of extensive graft versus host disease (GVHD).[40, 43, 44] Currently cord blood stem cells are used widely and reports has shown that the rate of engraftment is high and risk of severe GVHD is low.[45, 46] However, the number of HSCs that are present in a single unit of cord blood is not enough for transplantation to an adult patient, and more than one donor is needed.[47] Patient age, underlying disease and timing of transplant are important factors in determining type of transplant. Furthermore, the type and accessibility of donor may influence the transplant protocol used.

1.2.3 Major histocompatibility complex and types of HSCT

Prior to performing HSC, it is important to have an essential procedure done - MHC screening. The histocompatibility matching is one of the factors that play a major role in transplantation results. The MHC is a genetic locus with principal encoded proteins which present peptides to T cells. It plays important role in transplantation, immunity, inflammation, infections and different diseases.[48, 49]

Concerning the genetic difference between donor and recipients, HSCT could be divided into three types:

- Autologous, HSCs from the person himself, which have been preserved.
- Syngeneic, HSCs from an identical twin, i.e. they have similar major histocompatibility complex (MHC).
- Allogeneic, HSCs from a different MHC; this could be a sibling or a non-related donor.

In humans, MHC molecules are called human leukocyte antigens (HLA). The gene groups are present in chromosome number six. The complex region is divided conventionally into three classes; class I, II and III, each containing a cluster of genes with related functions.

Class IA and B proteins are found on the surface of nucleated cells in the body and present endogenous antigens to the cytotoxic CD8+ T cells,[50] while HLA-C is associated with natural killer (NK) antigen recognition. In the past, HLA-A and HLA-B were the only class I antigens routinely typed for transplantation, but it has recently been shown that differences at the HLA-C locus may be associated with higher rates of graft rejection and GVHD following unrelated donor stem cell transplantation.[51]

MHC class II are found on the antigen presenting cells such as B cells, monocytes and dendritic cells.[50] They present the exogenous antigens which are taken up by endocytosis or phagocytosis to helper CD4+ T cells. Class III genes encode the components of the complement system that target foreign cells and break their membranes.

The importance of matching patient and donor for HLA class I (A and B) and class II (DR) has long been established.[52-54] At present, evaluation of HLA typing using molecular techniques is mandatory.[55]

The patient's HLA type (HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1 and HLA-DPB1) is generally defined using DNA-based HLA typing techniques. These are based on PCR using sequence specific primers or sequence specific oligonucleotide probes.

HSCT with an HLA identical sibling donor is associated with the best transplant outcome.[56] However, the chances of finding a fully matched unrelated donor is about 30% to 70%.[57] Alternative related donors may be used with an increasing degree of HLA mismatching, including a haploidentical donor, which applies to 15% to 50% of patients.[58-60] The importance of MHC similarity and relationship between donor and recipient is clear from the lower risk of mortality and infection, the overall survival, and the decreased risk of GVHD.[61-63] Moreover, immune reconstitution occurs at an earlier time in patients with matched and related donors compared to in patients with mismatched donors.[64] However, a certain grade of MHC disparity is required when HSCT is used in malignancies where strong graft versus leukemic (GVL) effect is necessary.[65]

1.2.4 Conditioning and HSCT

Prior to carrying out HSCT it is essential that the patient is prepared by receiving conditioning agents. Generally, conditioning can be divided into two types according to the agent used, chemotherapy or total body irradiation (TBI). Furthermore, a combination of both is widely used. Sometimes other agents, for example anti-thymocyte, are added to the conditioning regimen.[66, 67] It has been shown that addition of anti-thymocyte to the conditioning regimen reduces graft failure.

The purpose of the conditioning is to provide a space for the new donor stem cells, to eliminate tumor and dysfunctional stem cells, and to suppress the immunity of the recipient in order to prevent rejection of the transplanted cells. All conditioning regimens, regardless of their intensity, induce some tissue damage which leads to inflammatory cytokine release.[68] Moreover, the residual host immune cells which survive after conditioning have an important effect on bone marrow transplantation (BMT) results and complications.[69, 70] The choice of conditioning protocol depends on different factors such as the underlying disease, the patient's age and the availability of facilities.[71, 72]

According to the intensity of the protocol, conditioning can be divided into two groups:

- Myeloablative regimens which are performed by using the maximum tolerable dose of the regimen used. This regimen results in severe pancytopenia within a few weeks from its start. The resulting pancytopenia is usually irreversible, and requires an infusion of hematopoietic stem cells for survival.

- Non-myeloablative regimens or reduced intensity regimens (RIC) cause reversible cytopenia. They have been used in clinics for more than a decade.[73]

RIC has been used for older patients.[74-76] Similarly RIC is starting to be used more in children.[77-79] Different studies in patients with acute myeloid leukemia have shown that transplant related mortality was decreased but relapse was increased in patients treated with RIC.[80] Reports have shown that the intensity of conditioning is important in the development of complications including GVHD.[81]

1.2.4.1 Total body irradiation (TBI)

The first conditioning regimen introduced to clinical HSCT was TBI.[35] TBI is an effective myeloablative treatment able to eradicate tumor cells. It has been used at low doses of less than one Gray (Gy) for various malignant disorders since 1900.[82] Even though the exact mechanism of action of radiation is not fully understood, it is suggested that the free radicals that are induced during the radiation process cause damages in the DNA chain, which finally leads to cell death. There is no exact optimal dose for TBI. Early myeloablative TBI regimens used single, large dose fractions of 8-10 Gy.[83-85] These kinds of regimens were accompanied by high risk of death from interstitial pneumonitis.[86, 87] It has been shown that fractionation and reduction of the dose reduce the risk of development of this complication.[87-89] Results from numerous studies showed that in both humans and rodents, dose rates of less than 10 to 12 cGy/min are associated with reduced rates of pneumonitis, nausea and vomiting.[88, 90, 91]

During radiation of the whole body, toxicity to every organ should be considered. However, it is difficult to assess the toxicity which is associated directly with TBI because other treatments such as antibiotics, antifungals, immunosuppressants, pain killers and chemotherapeutic agents are administered simultaneously during the BMT process.

Other main complications of TBI include secondary malignancy [92], cataracts [93, 94], growth retardation (especially in children) [95], veno-occlusive disease, interstitial pneumonitis, endocrine and central nervous system complications.[82]

In order to reduce the volume of total tissue exposed to radiation, blocks are used to protect different organs, e.g. lungs and eyes, without affecting the efficacy of treatment. Furthermore, cyclophosphamide (Cy) was added to the conditioning protocols in order to increase the immunosuppressive effect, decrease radiation toxicity and prevent graft rejection.[96]

1.2.4.2 Chemotherapy

Different chemotherapeutic agents have been used for conditioning procedure during preparation for HSCT. Busulfan (Bu), cyclophosphamide (Cy) and Fludarabine are widely used in addition to other agents such as chlorambucil, cytosine arabinoside, melphalan, thiotepa, carmustine, and etoposide.

Bu in combination with high-dose Cy is one of the most common conditioning regimens. It is a standard conditioning protocol for HSCT.[74]

Cy is an alkylating agent that belongs to the nitrogen mustard group. It is a pro-drug that is converted in the liver into active forms that have chemotherapeutic effects. It has been used in clinical practice since 1958 and is currently one of the most commonly used agents for treating various types of cancers and autoimmune disorders.[97, 98] Cyclophosphamide is converted to active metabolites by mixed-function oxidase enzymes in the liver [99] The main active metabolite is 4-hydroxycyclophosphamide, which exists in equilibrium with its tautomer, aldophosphamide. Most of the aldophosphamide is oxidized by the enzyme aldehyde dehydrogenase (ALDH) to make carboxyphosphamide. A small proportion of aldophosphamide is converted into phosphoramide mustard and acrolein. Acrolein is toxic to the bladder epithelium and can lead to hemorrhagic cystitis. This can be prevented through the use of aggressive hydration and/or mesna. The main effect of cyclophosphamide is due to its metabolite phosphoramide mustard. This metabolite is only formed in cells that have low levels of ALDH.[100, 101] However, it has been shown that it may also reduce the number of bone marrow stem cells and progenitor cells through damage to the stromal supportive cells.[102]

Bu is another alkylating agent that has reduced the use of TBI since its introduction to the BMT field. It has been used as a treatment for chronic myeloid leukemia.[103]

However, its use as a myelosuppressive agent in conditioning prior to HSCT was introduced in the 1990's.[104-107] Bu is given orally or intravenously. It displays a more marked effect on myeloid cells than on lymphoid cells. It is cytotoxic to hematopoietic stem cells. Prolonged aplasia may be seen after Bu administration.[102, 108]

Busulfan metabolism and detoxification as well as cyclophosphamide activation is mainly done in the liver.[97, 101, 109] Busulfan is metabolized primarily through the liver by conjugation to reduced glutathione (GSH). This process depletes hepatocyte glutathione stores. It has been shown that a high load of toxic metabolites and a shortage of protective components (e.g. GSH) during Bu-Cy conditioning induce hepatic toxicity and liver injury.[27, 110] A couple of studies concerned with altering the sequence of Bu and Cy administration have shown that this will give the same level of engraftment and also reduce the toxicity of the conditioning regimen.[111, 112]

1.2.5 Complications of HSCT

HSCT has become the standard treatment for many types of malignant and non-malignant disorders. Unfortunately, HSCT is often associated with severe complications. These complications can be acute (i.e. early) or late. Moreover, these complications may be due to the conditioning process or to the transplantation itself.

Infection due to different etiology is a very dangerous early complication. Deficiency in cellular and humoral aspects of the immune system occurs to some degree in every HSCT recipient. Usually recipients of allogeneic HSCT are highly susceptible to infections because of immunodeficiency, neutropenia, and immune suppressive therapy used to prevent or to treat GVHD. Different bacterial, viral and fungal infections can occur. The viruses that are regarded as most important in the HSCT setting include the herpes virus family, specifically cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpes virus 6, as well as human adenoviruses and the polyoma virus BK.[113-119]

Veno-occlusive disease (VOD) also known as sinusoidal obstructive syndrome (SOS) is another common complication. The symptoms of VOD start 1 to 4 weeks after conditioning. It occurs in 20 to 50% of patients receiving high dose TBI and those receiving high doses of cyclophosphamide and busulfan without TBI.[27, 120, 121] The risk factors include pre-transplantation elevation of serum aminotransferase, intensive conditioning therapy, graft from mismatched or unrelated donor and use of antimicrobial therapy with Acyclovir, amphotericin B, or Vancomycin.[122] The pathogenesis behind VOD development is not well known. It is thought that the obstruction of the venules by endothelial cell injury and thrombosis is the main pathology behind this condition.

Graft failure is another early or late complication after allogeneic HSCT. It is evidenced by lack of early or late hematopoietic recovery in association with recurrence of the disease or reappearance of the host cells after initial donor cell engraftment. The incidence of marrow engraftment failure is rare, i.e. less than 2% in patients with hematologic malignancies receiving complete HLA identical marrow. The complication is more commonly seen in patients with aplastic anemia receiving allogeneic BMT.[123]

Several late complications can occur after HSCT. These include endocrine complications.[124] Hypo- or hyperthyroidism could occur.[125, 126] Growth hormone deficiency has been reported, mainly in children conditioned with high dose TBI.[127] Impaired sexual maturity and infertility are common complications in those treated with TBI,[128] and there is also a high incidence of cataracts.[129, 130] Neurological toxic effects include leukoencephalopathy, cerebral ataxia, and seizures as possible late complications. This is especially the case in children receiving irradiation or intrathecal chemotherapy.[131-133]

Aseptic necrosis of the hips has been reported after HSCT.[134, 135] There is also an increased incidence of secondary malignancies.[136] Other complications include respiratory failure from multiple etiologies, GVHD, multi organ failure, hemorrhagic cystitis and mucositis.[137-141]

1.2.6 Graft versus host disease (GVHD)

GVHD is a one of the major complication after allogeneic HSCT.[142] The development of GVHD can be divided into acute, which occurs within the first 100 days after transplantation, and chronic, which starts after 100 days.[143] However, the mechanism and the pathological features of these two entities are different. Moreover, acute GVHD can be developed beyond 100 days in patients transplanted using non myeloablative conditioning.[144, 145] The incidence of acute or chronic GVHD fluctuates between different centers; however, acute GVHD develops in 40% to 70% of all allogeneic-transplanted patients while chronic GVHD may occur in 28% to 100% of patients.[146]

During GVHD, immune cells from the donor identify the host environment as a foreign environment and start to attack different tissues in the recipient leading to development of fatal symptoms.[147, 148] The donor T cells are among the cells responsible for acute GVHD; CD4+ and CD8+ αβ T cells are reported to be the main inducers of GVHD by different mechanisms.[149-152]

Acute GVHD is characterized by dermatitis, hepatitis and enteritis. A maculopapular rash involving the trunk, face, extremities, palms, soles of the feet and ears usually characterizes the onset of acute GVHD. The liver function tests including alkaline phosphatase, and aminotransferase levels increase in addition to increased levels of bilirubin. These signs are usually accompanied by gastrointestinal symptoms such as nausea, vomiting, abdominal pain and massive watery or bloody diarrhea. Acute GVHD is graded as I-IV based on severity of involvement of the target organs for GVHD such as skin, liver, and gastrointestinal tract.

It is presumed that conditioning induces tissue damage and subsequent lipopolysaccharide (LPS) extravasation from the damage to the gastrointestinal tract (GIT), promotes inflammation and cytokine storm and leads to activation of donor T cells by host/donor APCs.[147, 148, 153, 154]

However, three basic components for developing GVHD have been suggested by Billingham.[155] First, the graft should contain immune competent cells, mostly donor T lymphocytes. [156, 157] Second, the recipient must have low immunity and be unable to reject transplanted cells and third, the recipient tissues must present antigens that are not expressed by the donor’s cells.[158]

Acute GVHD occurs as a consequence of donor T cell recognizing the recipient HLA antigens as foreign, starting to attack tissues of the host and developing signs and symptoms of acute GVHD.[143] The tissue damage occurring in acute GVHD may be mediated by infiltration of the target organ by natural killer lymphocytes. Interleukin 1 (IL-1), tumor necrosis factor (TNFα), and IL-2 are thought to be critical to the development of acute GVHD.[159]

Different studies reported that GVHD develops in three sequential stages. ***i)*** Inflammation and cytokine storm as result of pre-transplant conditioning. ***ii)*** Activation of donor T cells through recipient/donor APC and ***iii)*** Damage of certain tissues by the activated donor T cells.[153, 160-167] Intestine, skin, and liver are the organs most frequently affected by allo-reactive donor T cells in the GVHD process.[168]

Investigations have shown that the occurrence and severity of GVHD depend on several factors including: intensity of pre-transplant conditioning, presence and number of donor T cells, antigenic difference between donor and recipient with a high risk in HLA mismatch and unrelated HLA identical donor [81, 163, 169, 170], female donor to male recipient (previous donor pregnancies), increase in donor or recipient age, unsuccessful GVHD prophylaxis and previous recipient infections with herpes viruses. However, GVHD may occur in any type of allogeneic setting regardless of conditioning protocol.[144, 171]

1.3 CHRONIC KIDNEY DISEASE (CKD) AND IMMUNITY

CKD is the slow loss of kidney function over time. It is a general term used to describe a wide scope of disorders affecting the function and histology of the kidney. The major causes are glomerulonephritis, diabetes mellitus, nephrosclerosis, interstitial nephritis, and polycystic kidney disease. It is usually associated with diabetes, hypertension, obesity, and old age in developed countries. However, the exact pathology is unknown.[172]

For defining CKD, we depend on the presence of albuminuria or decreased renal function, i.e. a glomerular filtration rate (GFR) of less than 60 ml/min per 1.73m^2 for three months or, more independently, on the appearance of clinical signs.

Kidney function can be measured either directly by measuring the GFR [173, 174], or indirectly by determining the concentration of creatinine in the serum. Measuring creatinine concentration in serum is fast and simple, and it is the most commonly used screening test for renal function; however, as much as 50% of the nephrons may be lost before the creatinine levels increase. Furthermore; the levels of creatinine are influenced by extra-renal elimination, muscular mass, body mass, age and diet.[175]

The National Kidney Foundation in the United States classified CKD into five stages based on the level of GFR. Stage I denotes normal or elevated GFR (more than 90 ml/min per 1.73 m^2), stage II is 60 to 89 ml/min per 1.73 m^2, stage III is 30 to 59 ml/min per 1.73 m^2, stage IV is 15 to 29 ml/min per 1.73 m^2, and stage V represents a GFR of less than 15 ml/min per 1.73 m^2 or treatment with dialysis and this is called end stage renal failure.

When end stage renal disease is developed, patients are in increased risk of susceptibility to infection complications.[176-178] Impairment of defense mechanisms is responsible for the increased susceptibility; however, dysfunctional polymorphonuclear cells are a contributing factor.[179] This dysfunctionality is exhibited as reduced chemotaxis, decreased phagocytic ability, reduced intracellular killing and increased apoptosis rate.[180, 181] In addition, these patients display extended survival of skin allograft, reduced immunization against hepatitis B, diphtheria, influenza, and tetanus vaccine, which indicates immune defects in antigen presenting cells.[182-186] Malnutrition, loss of vitamins, iron overload, and uremic toxins also contribute to leukocyte dysfunction.[179, 187] CKD is associated with an acquired immune deficiency concerning both cellular and humoral immunity.

These patients have been identified as being in a state of low grade inflammation, which renders them less responsive.[188] The state of immune dysfunction that occurs in uremia can be characterized by the presence of both immunoactivation and immunodepression.[189] Despite the increased secretion of proinflammatory cytokines, both monocytes and monocyte-derived cells might have a reduced antigen presentation capacity in patients on chronic hemodialysis.[189] The impaired expression of co-stimulatory molecules also contributes to this phenomenon.[190]

Studies in patients on dialysis showed that reduced renal clearance of cytokines might contribute to elevated cytokine levels in peripheral blood.[191] Importantly, an increase in plasma levels of proinflammatory cytokines is associated with mortality in patients on dialysis.[192]

In addition, different studies have reported an increased risk of accelerated atherosclerosis in CKD.[193-195] Atherosclerosis is an inflammatory disease which attributes a pivotal pathophysiological role to monocytes and macrophages. Monocytes are considered instrumental in the development of atherosclerosis from the earliest morphological lesions until final plaque rupture.

2 AIMS

2.1 GENERAL AIMS

The overall aim of this thesis is to investigate changes in gene expression during conditions accompanied by disturbance in immune system, with a special concern to hematopoietic stem cell transplantation and chronic kidney disease. The goal is to gain insight into the mechanisms responsible for these conditions and to identify new pathways that can contribute to the development of new treatment strategies and give further understanding of the pathophysiology of the disease.

2.2 SPECIFIC AIMS

- To study the gene expression profile early after HSCT in target organs and non-target organs of acute GVHD, and to find out whether specific pathways characterize target and non-target organs.

- To define the role of expression of PD-L1/CD274 in different organs early after HSCT and to understand the importance of this expression in protecting non-target organs from GVHD.

- To evaluate the gene expression profile and hence to assess immunological changes that accompany different strategies used for delivering TBI, either single dose or fractionated, during conditioning before hematopoietic stem cell transplantation.

- To investigate the gene expression profile of monocytes from CKD patients in comparison to healthy persons and to find activated pathways in this patient group. This may help us understand the pathophysiology behind the monocyte dysfunction.

3 MATERIALS AND METHODS

3.1 ANIMALS

In papers I, II, and III, female BALB/c (H-2Kd) and male C57BL/6 (H-2Kb) mice obtained from Scanbur (Sollentuna, Sweden) were used. The mice were 10 to 14 weeks old at the time of the experiments. Animals were kept in individually ventilated cages and maintained under specific pathogen free conditions in an animal facility with controlled humidity (55% ± 5%), 12 hours light/dark cycle, controlled temperature (21ºC ± 2ºC) and HEPA filtered air. The animals were fed autoclaved mouse chow and tap water *ad libitum*, and were allowed to acclimatize to their surroundings for one week before the start of the experiments. All animal experiments were approved by the Stockholm southern ethical committee and in accordance with the Animal Protection Law, the Animal Protection Regulation and the regulation of the Swedish National Board for Laboratory Animals.

For bone marrow transplantation in the syngeneic setting, female BALB/c (H-2Kd) mice were used as recipients and female BALB/c mice were used as donors, while in the allogeneic setting female BALB/c mice were used as recipients and male C57Bl/6 (H-2Kb) mice were used as donors. Untreated mice were used as controls whenever appropriate.

3.2 CONDITIONING

The chemotherapy conditioning for paper I and II consisted of Bu 20 mg/kg/day for four consecutive days (day -7 to day -4) followed by Cy 100 mg/kg/day for two days (day -3 and day -2). This six day regimen was followed by one day of rest (day -1) and then bone marrow transplantation the day after (day 0). Both Bu and Cy were purchased from Sigma-Aldrich (Sigma-Aldrich Stockholm, Sweden). Both drugs were given through intra-peritoneal injection. For irradiation-based conditioning in paper III, mice received total body irradiation of 850 cGy either as a single dose or two fractionated doses employing a cesium source (Gammacell® 40 Exactor, Best Theratronics, Ottawa, Ontario, Canada).

3.3 BONE MARROW TRANSPLANTATION

For preparing bone marrow the donor mice (either allogeneic or syngeneic setting) were sacrificed by cervical dislocation. Bone marrow cells (BMC) were flushed from both femurs and tibias. Single-cell suspension was prepared by passing cells gently through a 14G needle in RPMI-1640 containing 2% fetal bovine serum (FBS). The cells were then centrifuged and washed with RPMI. Donor spleens were disrupted in RPMI-containing 2% FBS to form a single cell suspension. Splenocytes were passed through a 70 µm strainer, centrifuged and washed two times with RPMI. Cell number and viability was measured using Trypan blue exclusion dye. Recipient mice received 2 x 10^7 BMC in combination with 3 x 10^7 splenocytes intravenously through the lateral tail vein in a volume of 200 µl in PBS (Phosphate buffer saline).

3.4 TISSUE PREPARATION

In all experiments involving mice, the entire mouse body was perfused through the heart with 20 to 30 ml ice cold PBS containing 2% FBS 10mM EDTA until the organs became pale in order to exclude any confusing effects of circulatory cells in tissues. Parts of the tissue were then snap frozen in liquid nitrogen and transferred to -150 ºC until used.

3.5 ASSESMENT OF GVHD

Recipient mice were checked daily from the start day of conditioning until the day of taking samples. They were evaluated for five signs: weight loss, posture, activity, fur texture and skin integrity. These signs represent signs of acute GVHD in mice. The severity of each given symptom was scored from 0 to 2. The sum of the scores for all symptoms in each mouse (maximally 10) was used as an index of the severity and progression of GVHD.

3.6 RNA PURIFICATION

Total RNA from frozen sections was extracted with the Qiagen RNeasy kit (WVR, Stockholm, Sweden) according to the manufacturer's instructions. The integrity of extracted RNA was confirmed using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) with extracts exhibiting absorbance in a ratio of 1.90 to 2.0 at 260/280 nm being regarded as of acceptable purity, and agarose gel was run to confirm purity of RNA.

3.7 CDNA SYNTHESIS FOR MICROARRAY ANALYSIS

3.7.1 Affymetrix

Double-stranded complementary DNA (cDNA) was synthesized with 50 ng of total RNA using the SuperScript Choice system (Invitrogen Inc). T7-(dT24) oligomer was used for priming the first-strand cDNA synthesis. The resultant cDNA was purified using the Sample clean up kit (Affymetrix Inc). The cDNA pellet was collected and dissolved in an appropriate volume. Using cDNA as the template, cRNA was synthesized using an In-vitro transcription (IVT) kit (Affymetrix Inc). IVT reactions were carried out at 37°C for 16 hours and the labeled cRNA was purified using the Sample clean up kit (Affymetrix Inc). The cRNA was fragmented in a fragmentation buffer (40 mmol/l Tris-acetate, pH 8.1, 100 mmol/l KOAc, 30 mmol/l MgOAc) for 35 min at 94°C. Fragmented cRNA (15 µg/probe array) was hybridized with Mouse Genome 430 2.0 GeneChip arrays (Paper I) or Human U133A GeneChip arrays (Paper IV) at 45°C for 18 hours in a hybridization oven with constant rotation (60 rpm). The chips were washed and stained using the Affymetrix fluidics station. Staining was performed using Streptavidin phycoerythrin conjugate (SAPE; Molecular Probes, Eugene, OR, USA), followed by the addition of a biotinylated anti-Streptavidin antibody (Vector Laboratories, Burlingame, CA, USA), and finally with Streptavidin phycoerythrin conjugate. Probe arrays were scanned using fluorometric scanners (Affymetrix Scanner). The scanned images were visualized and analyzed using established quality control criteria.

3.7.2 NimbleGen

Double stranded cDNA was prepared from 10 µg total RNA as a starting material, using the Invitrogen Superscript Double-Stranded cDNA Synthesis Kit. Clean up of the cDNA was done. Then 1µg of cDNA was used in labeling with Cy3 by NimbleGen One-Color DNA Labeling Kit. 4µg of labeled cDNA was used for hybridization to a NimbleGen array (12 plexus gene array was used, 12x135K (12 x 132,510 probes equal to 44170 genes)) using a NimbleGen Hybridization System. Subsequent steps include washing, drying, and scanning the array with the MS 200 Microarray Scanner and the MS 200 DATA Collection Software.

3.8 REAL-TIME PCR (RT-PCR)

Total RNA was used to synthesize single strand cDNA by using Superscript II reverse transcriptase system and oligo dT12-18 according to manufacturer's instructions (Invitrogen Inc., CA, USA). Using cDNA as a template, TaqMan gene expression assays were performed by means of the FAM dye labeling system according to the manufacturer's instructions (Applied Biosystems, Stockholm, Sweden). TaqMan assay for selected genes and the house keeping gene β-actin (Actb) were used for detection of genes (Applied Biosystems, Stockholm, Sweden).

RT-PCR reactions were performed using a 384 well plate in the ABI 7900 Thermal cycler (Applied Biosystems, Stockholm, Sweden). The results were normalized against the house keeping gene Actb by subtracting the Ct value of house-keeping gene from Ct of the specific gene (Ct gene- Ct Actb) giving a delta Ct (ΔCt) value. Relative gene expression was calculated using the equation 2 -ΔCt. The value of ΔΔCt for an individual gene, when comparing day of interest to control, was calculated by subtracting ΔCt of control from ΔCt of specific time point (ΔCt specific day -ΔCt of control) and the fold change was calculated using the equation 2 -ΔΔCt.

3.9 IMMUNOHISTOPATHOLOGY

Tissue samples were fixed in neutral buffered formalin for 24 hours, transferred to 70% ethanol, dehydrated and embedded in paraffin according to standard procedures. Sections of 4µm were prepared, mounted on glass slides and routinely stained with H&E or prepared for immunohistochemistry.

After rehydration in a graded alcohol series and antigen retrieval, sections were treated with 0.3% H_2O_2 for immunohistochemistry staining for PD-L1/CD274 (Paper II) to block endogenous peroxidase activity. After blocking with 10% goat serum, samples were incubated overnight with goat anti-mouse PD-L1 (R and D Systems) and diluted in 2% bovine serum albumin/phosphate-buffered saline as the primary antibody. After washing, sections were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (ZYMED Lab, Invitrogen), at 1:500 dilution. Sections were developed using the HRP-DAB system (R and D Systems), counterstained with Mayer's hematoxylin (Sigma), dehydrated through a graded alcohol series, and cover slipped.

3.10 PATIENTS

Patients with renal failure were recruited from the Department of Nephrology at Danderyd Hospital. All participants gave their written informed consent and the study was approved by the Ethics committee of the Karolinska Hospital. All patients and healthy subjects suffering from infectious diseases, diabetes mellitus or active inflammatory diseases as well as those receiving antibiotics, corticosteroids or non-steroidal anti-inflammatory agents were excluded from the study.

The study population consisted of fourteen patients with severe renal failure (GFR less than 20 ml/min per 1.73 m^2 according to the Cockcroft and Gault equation) and ten healthy subjects. The healthy subjects were age and sex matched with the patients. The renal diagnoses were the following; nephrosclerosis, polycystic kidney disease and renal failure of unknown origin.

3.11 MONOCYTES (COLLECTION & PURIFICATION)

50-60 ml of peripheral blood was collected in 9 ml tubes containing 135 USP U sodium heparin (Venosafe, Terumo Europe, Leuven, Belgium). Monocytes were isolated from the peripheral blood by density centrifugation. Briefly, blood was diluted 1:1 with RPMI 1640. The diluted blood was then layered on 25ml Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 400 ^{x}g in room temperature for 30 minutes. The monocyte rich white color band was collected. A positive selection of monocytes was performed by incubating the monocytes with anti-CD14 coupled to MACS beads (Miltenyi Biotec, Auburn, California, USA). The cells were then loaded onto a MidiMACS column and the CD14 positive monocytes were collected.

3.12 PURITY OF MONOCYTES BY FLOW CYTOMETRY

The purity of monocyte fraction was analyzed by flow cytometry (Beckman Coulter Inc., Hialeah, Florida, USA). This instrument gives the actual number of cells and the mean fluorescence intensity (MFI). Monocyte cell populations were distinguished by their different light scattering properties (Forward Scatter (FS)/Side Scatter (SS)). The forward scatter signal (y-axis) reflects the cell size while the side scatter signal (x-axis) reflects cell granularity. The percentage and absolute number of positively immunostained monocytes was determined by measuring MFI of the positive cell population after staining with CD14–ECD (Beckman Coulter, Marseille, France) and using isotype mouse IgG2 (Beckman Coulter, Marseille, France). Only fractions above 95% purity were used for further experiments.

3.13 MEASURING TNF-α AND IFN-γ BY SANDWICH ELISA

At different time points before and after transplantation, the mice were bled by retro-orbital puncture under light isoflurane anesthesia and thereafter sacrificed by cervical dislocation. The blood samples were centrifuged to obtain serum, which was stored at −80°C until analysis. The levels of TNF-α and IFN-γ in the sera were quantified employing commercially available ELISA kits (eBiosciense, San Diego, CA, USA), in accordance with the manufacturer's instructions.

3.14 WESTERN BLOT

Lysate for western blot was prepared in the following manner: for paper IV, whole cell lysate was used, and for paper II, 10-20 mg frozen samples of muscle, liver and kidney from different time points were used.

Isolated purified monocytes were washed with cold PBS and lysed in modified radioimmunoprecipitation assay buffer (modified RIPA). Two cycles of freezing and thawing on dry ice with ethanol were completed, and then the sample was left in ice for 30 min. Debris was removed by centrifugation at 10000 rpm for 10 min at 4ºC and the supernatant was collected.

Tissues samples were finely minced with a lancet and subjected to interrupted cycles of sonication. Using the probe sonication, the instrument was adjusted to a 0.5 cycle and the cycle amplitude to 50 kHz. Samples were subjected to 10 short bursts of 10 second duration followed by intervals of 30 seconds for cooling, and the procedure was repeated several times until complete lysis was achieved. The lysates were centrifuged at 16 000 rpm for 15 minutes at 4ºC and the supernatant was collected. Measuring of protein concentration was done by special available commercial kits.

Protein samples were then stored in sample buffer containing 0.0625M Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 0.025% bromophenol blue.

The lysates in sample buffer were boiled at 95°C for 5 minutes and separated by special gels. Gels were blotted on a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA) or nitrocellulose membranes. The membranes were blocked in 5% milk followed by incubation with primary antibodies overnight. After washing, the membranes were incubated with secondary antibody conjugated horseradish peroxidase followed by enhanced chemiluminescence (ECL) detection (ECL Plus, Amersham Pharmacia, Uppsala, Sweden) for paper IV, or (for paper II) incubated with an IRDye ® 800CW conjugated secondary antibody (LI-COR, Nebraska, USA) followed by visualization of the proteins using the specialized imaging system.

3.15 MICROARRAY DATA ANALYSIS SOFT WARE

Data analysis was performed using Affymetrix GeneChip Operating Software (GCOS). A quantitative signal and a qualitative detection call were generated for each sample and transcript. Data files were subsequently analyzed utilizing the Gene Spring GX (Silicon Genetics, CA, USA)

3.16 GENE EXPRESSION PATHWAY ANALYSIS SOFTWARE

Enrichment analysis was performed using the online gene set analysis toolkit WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) and Ingenuity pathway analysis for paper I. For other papers Panther tool were used.

3.17 STATISTICAL ANALYSIS

ANOVA test was used in paper II and III. Unpaired t-test was used for analysis of microarray results for paper IV. All the data are presented as mean ± SE.

4 RESULTS

4.1 PAPER I

4.1.1 Establishment of acute GVHD

Using different strains of donor and recipient mice, C57BL/6 as donors and BALB/c as recipient, induces lethal acute GVHD. The preliminary clinical signs of acute GVHD appear one week after allogeneic HSCT. Clinical indications of GVHD (weight loss, hunched posture, poor activity, ruffled fur and loss of skin integrity) were observed in allogeneic-transplanted animals while the syngeneic group recovered to normal health conditions.

Histological findings at day twenty one post transplantation in the skin and intestine of the allogeneic group confirmed the occurrence of GVHD while syngeneic-transplanted animals were identical to untreated mice (Figure 2).

4.1.2 Gene expression after chemotherapy conditioning

Expressions of 164, 114 and 660 genes were changed in the liver, kidney and muscle, respectively, after chemotherapy conditioning (day 0).

Genes involved in hemoglobin synthesis exclusively decreased in all tissues. Reciprocally, expression levels of only two genes, FK506-binding protein 5 and Vanin1, increased in all studied tissues after conditioning.

Inflammation-related genes like CXCL1, IFNγ-induced GTPase (Igtp), signal transducers and activators of transcription (STAT3) as well as intercellular adhesion molecule 1 (ICAM-1) were up regulated by 13, 2.8, 2.3 and 2.1 fold, respectively, in the liver, while not changing or decreasing in the kidney or muscle.

JAK-STAT signaling pathway was significantly ($P<0.0006$) over expressed in the liver while no other tissues showed this pattern (Figure 3). Genes involved in this pathway, e.g. SOCS3, STAT3 and Cyclin D1, were significantly over expressed by 2.8, 2.3 and 2.6 respectively in the liver after chemotherapy conditioning.

Pathway analysis of over expressed genes in the kidney or muscle did not result in any biological processes.

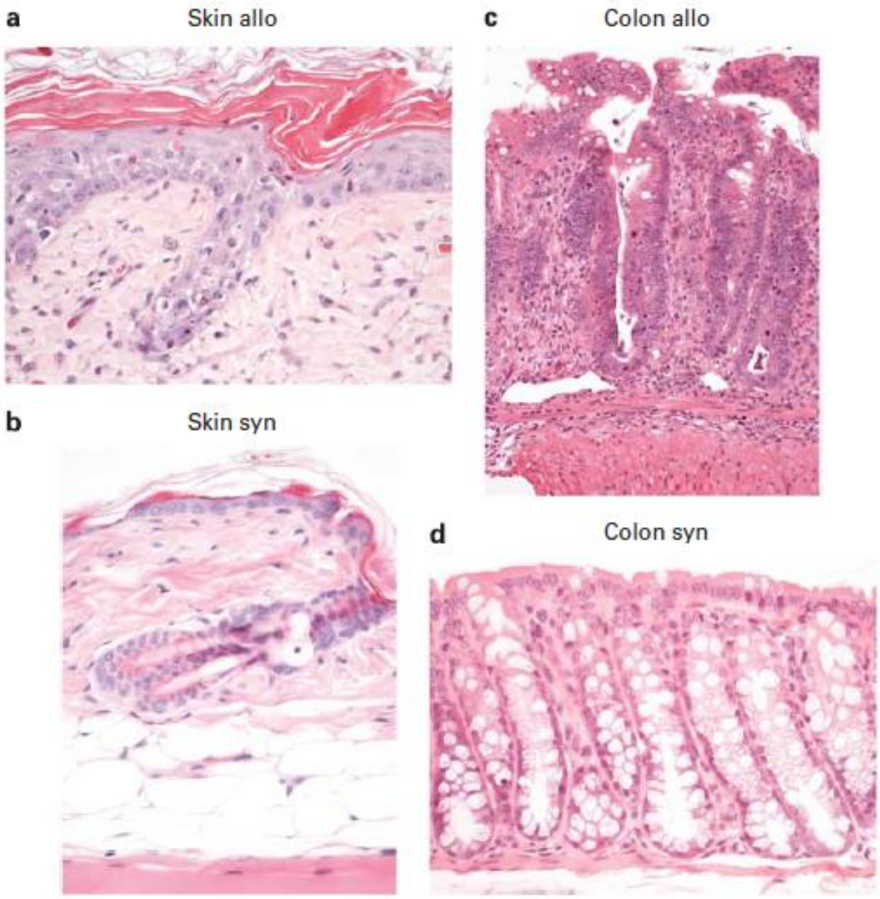

Figure 2. Development of GVHD in allogeneic-transplanted mice versus syngeneic-transplanted mice on day 21 after transplantation: (a) Skin of allogeneic-transplanted mice; (b) skin of syngeneic-transplanted mice; (c) intestine of allogeneic-transplanted mice; (d) intestine of syngeneic-transplanted mice

4.1.3 Allogeneicity and inflammatory genes

4.1.3.1 Liver

621 and 590 genes totally were up and down regulated respectively in the liver of GVHD mice as compared to syngeneic-transplanted mice. A majority of over expressed genes in the liver of GVHD mice were related to immune response and inflammation. Genes which are important in antigen presentation, leukocyte migration and activation, were significantly up regulated in the liver of GVHD mice (P<0.001).

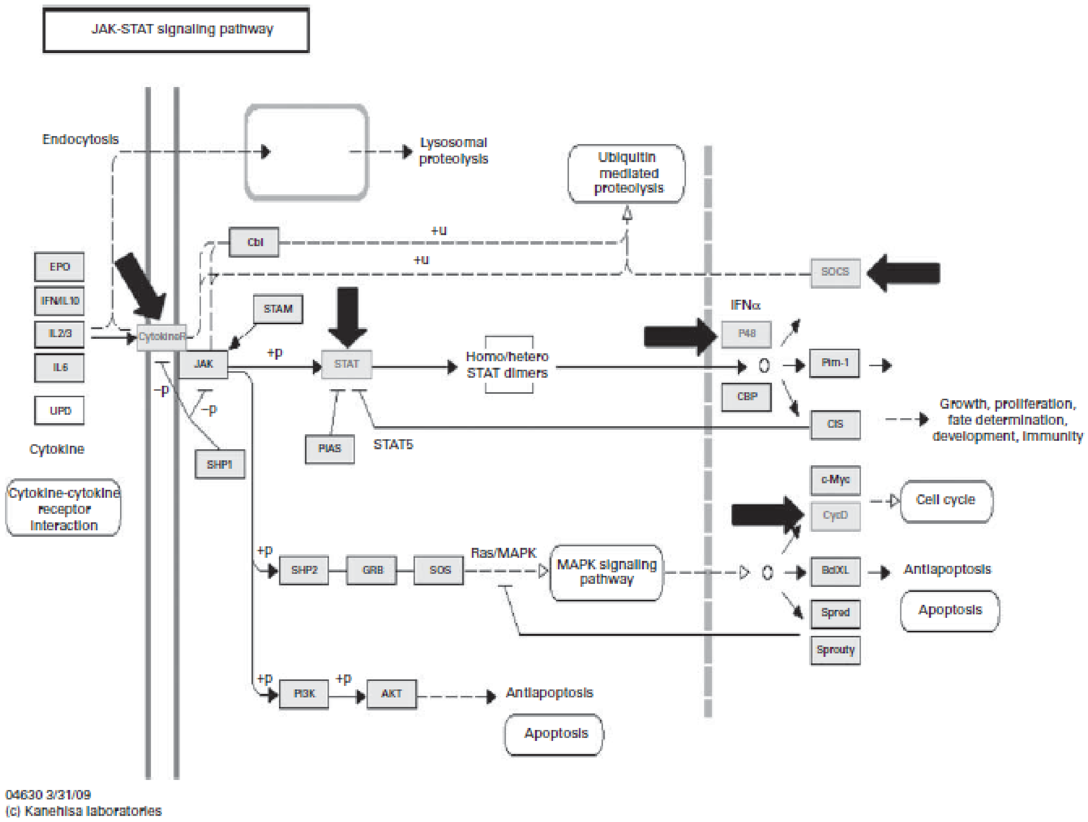

Figure 3. JAK–STAT pathway in the liver. Arrows indicate over expressed genes.

Toll-like receptor signaling (P<0.0026) and graft versus host disease (P<=9.28e-09) pathways were significantly provoked in the liver of GVHD mice, and this appropriately explains the pathophysiology of liver damage during GVHD. Interestingly, CD14 (22.7 FC), LBP (5.3 FC), TLR1 (5.3 FC) and TLR2 (7 FC) genes (targets for bacterial invasion and LPS stimulation) were significantly up regulated in liver tissue. None of these genes, biological processes or pathways increased in the liver of syngeneic-transplanted mice. These findings highlight the role of the innate immune system and importance of LPS extravasations in the pathogenesis of GVHD in the liver.

4.1.3.2 Kidney

The gene expression profile showed that numerous genes related to inflammation and immune response were up regulated in kidneys from mice developing GVHD while remaining unchanged or even decreasing in kidneys from syngeneic-transplanted mice.

The genes Chitinase 3-like 3 (955 fold), CXCL9 (72 fold), IFN-inducible GTPase 1 (72 fold), IFNγ-inducible protein 47 (49 fold), Histocompatibility 2, Q region (49 fold), Histocompatibility 2, class II, locus Mb2 (49 fold), Macrophage activation-2 like (45 fold), Granzyme B (39 fold), Granzyme -K (34 fold), CD3 Ag, γ-polypeptide (34 fold) and CXCL11 (32 fold), which directly or indirectly are relevant to immune response and/or inflammation, were significantly up regulated in the kidneys of GVHD mice. Genes involved in antigen processing and presentation were highly expressed.

4.1.3.3 Muscle

In the muscle of GVHD mice, 3918 genes were either up or down regulated as compared to the syngeneic group. Among them, several inflammatory genes which mainly relate to systemic inflammation and cytokine effects were over expressed in the GVHD group. Although the majority of the pathways that involved up regulated genes (more than 70%) belonged to cell biological and metabolic clusters, less than 30% of them directed to antigen processing and presentation. Contrasting liver and kidney, the important genes for chemotaxis like IL-1-β, chemokines and adhesive molecules e.g. CXCL9, CXCL1, CCL1, Saa1, Saa3, STAT1 and VCAM either were not expressed or were significantly less expressed in the muscle. Thus, despite muscle of GVHD mice presenting more inflammatory genes than muscle from syngeneic-transplanted animals, they were still expressing much less inflammatory, chemotactic genes compared with the liver and kidneys of GVHD mice.

4.1.4 Gene expression in target organs

There was high up regulation in the expression of genes involved in immunological and inflammatory response in the liver and kidney compared to muscle in GVHD mice (Figure 4a). Moreover, the majority of the inflammatory related biological and molecular processes were up regulated in the liver or kidney but not in the muscle (Figure 4b).

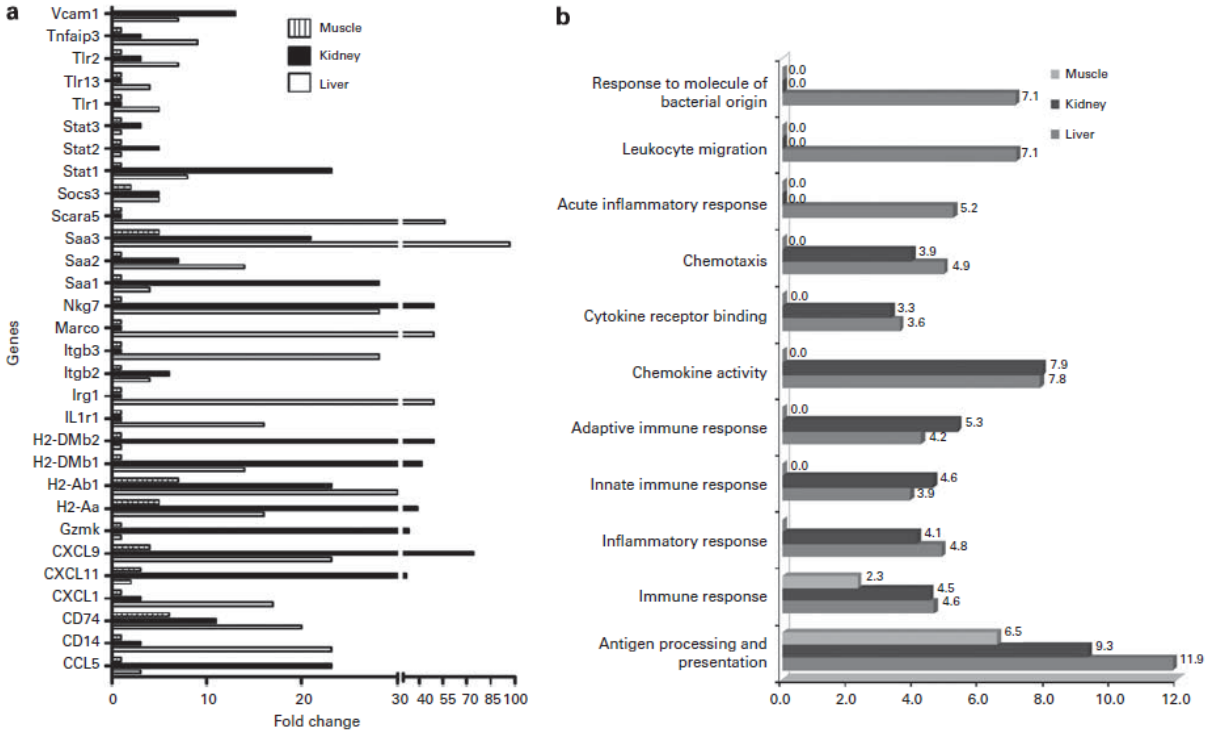

Figure 4. Up regulated genes and biological process in target tissues versus non-target tissues in GVHD-developing mice.

4.1.5 The kidney could be a target organ for acute GVHD

311 genes were exclusively up regulated in the kidney of allogeneic-transplanted mice. These genes were unchanged or even decreased in kidneys from syngeneic-transplanted animals and also muscle from GVHD mice. Most of these genes were related to antigen presenting and immune response. The over expressed genes and activated biological processes in the kidney are comparable to those observed in the liver (target tissue for GVHD) and are significantly different from those observed in muscle from GVHD mice (Figure 4). Furthermore, exploration of CD3+ T lymphocytes were confirmed in kidneys from allogeneic-transplanted mice but not in syngeneic-transplanted mice at day 21 after transplantation, which is similar to the results from the liver of mice transplanted with allogeneic bone marrow at the same time (Figure 5).

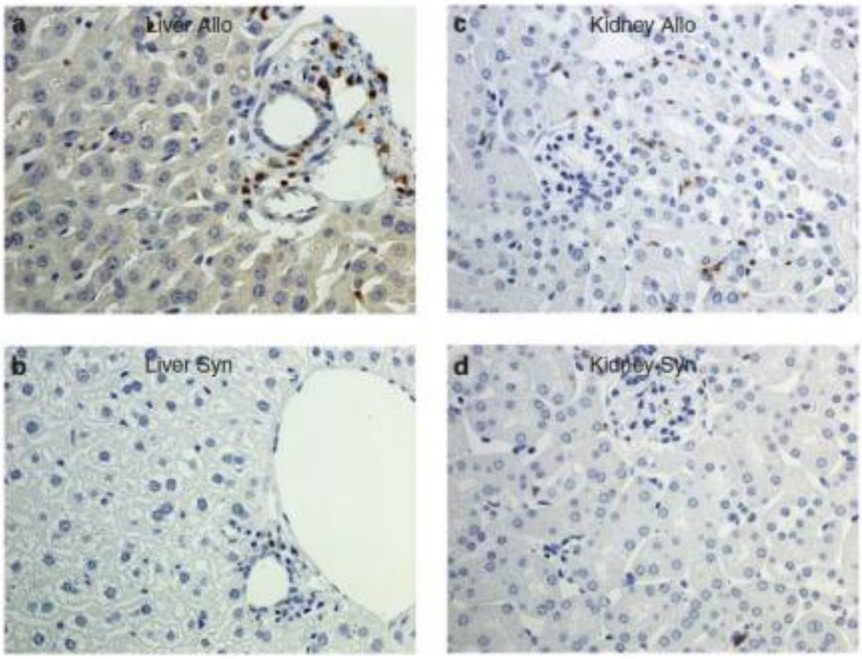

Figure 5. Infiltration of CD3+ T cells in the kidney of GVHD mice. (a) The liver, allogeneic (GVHD) group (b) the liver, syngeneic group (c) the kidney, allogeneic (GVHD) group (d) the kidney, syngeneic group.

4.2 PAPER II

4.2.1 PD-L1/CD274 in muscle, liver, and kidney

In untreated animals, the expression of PD-L1/CD274 mRNA was significantly higher in muscle compared to liver ($p<0.05$). Furthermore, the expression of CD274 after conditioning (i.e. at D0) was increased by ten folds in muscle tissue compared to before conditioning D-7, whereas the increase was minimal in the liver and the kidney (Figure 6).

4.2.2 PD-L1 in target and non-target organs

At the time of transplantation (D0) both syngeneic- and allogeneic-transplanted animals exhibited an increase in the expression of PD-L1 in all tested organs (Figure 7a-c). The syngeneic transplanted group showed a gradual decline in the expression of PD-L1 in all organs, starting on D+1 post transplantation. In contrast, the allogeneic-transplanted group exhibited a continued significant ($p<0.05$) increase in the expression of this molecule, which peaked at day +5 (muscle and kidney) or +7 (liver) and then gradually declined without reaching control levels even on day 21 post transplantation (Figure 7a-c).

4.2.3 Higher PD-L1 is in non-target organs

On day +5, the fold-increase in the expression of PD-L1 in the muscle was significantly higher (p<0.01) than that in the kidney (28-fold) and liver (95-fold) (Figure 8a). The expression of PD-L1 was still significantly higher (p<0.05) in the muscle than in the kidney and liver 7 days after allogeneic transplantation, as measured by both qPCR and microarray (Figure 8b).

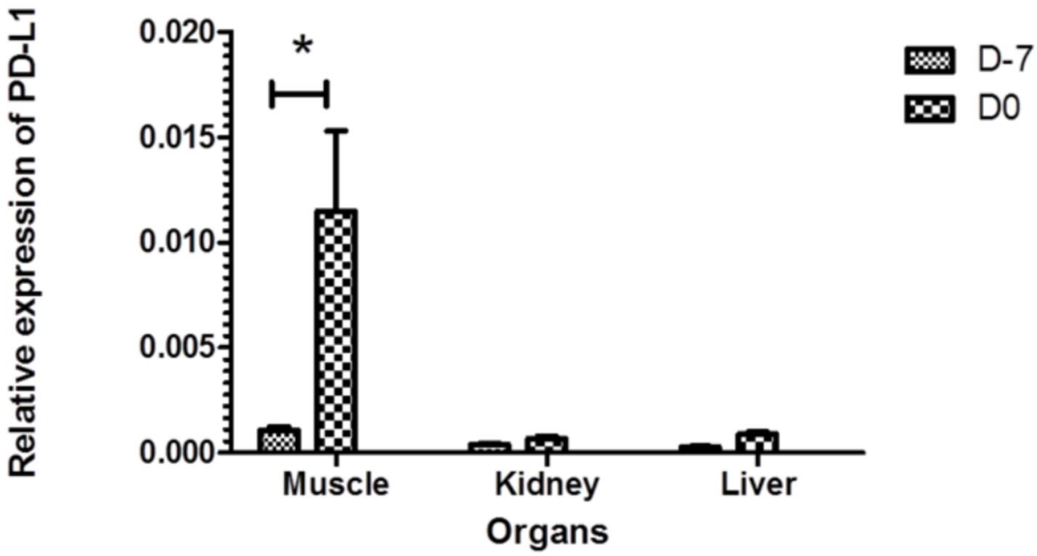

Figure 6. PD-L1/CD274 mRNA expression in different organs in control (D-7) and conditioned (D0) mice. Samples from control mice (D-7) and conditioned mice (D0) were collected, mRNA was prepared and qPCR was performed. CD274 expression was calculated as a ratio to the house keeping gene (β actin; ACTB). The results are presented as mean ± SE. * significant difference (*p< 0.05*)

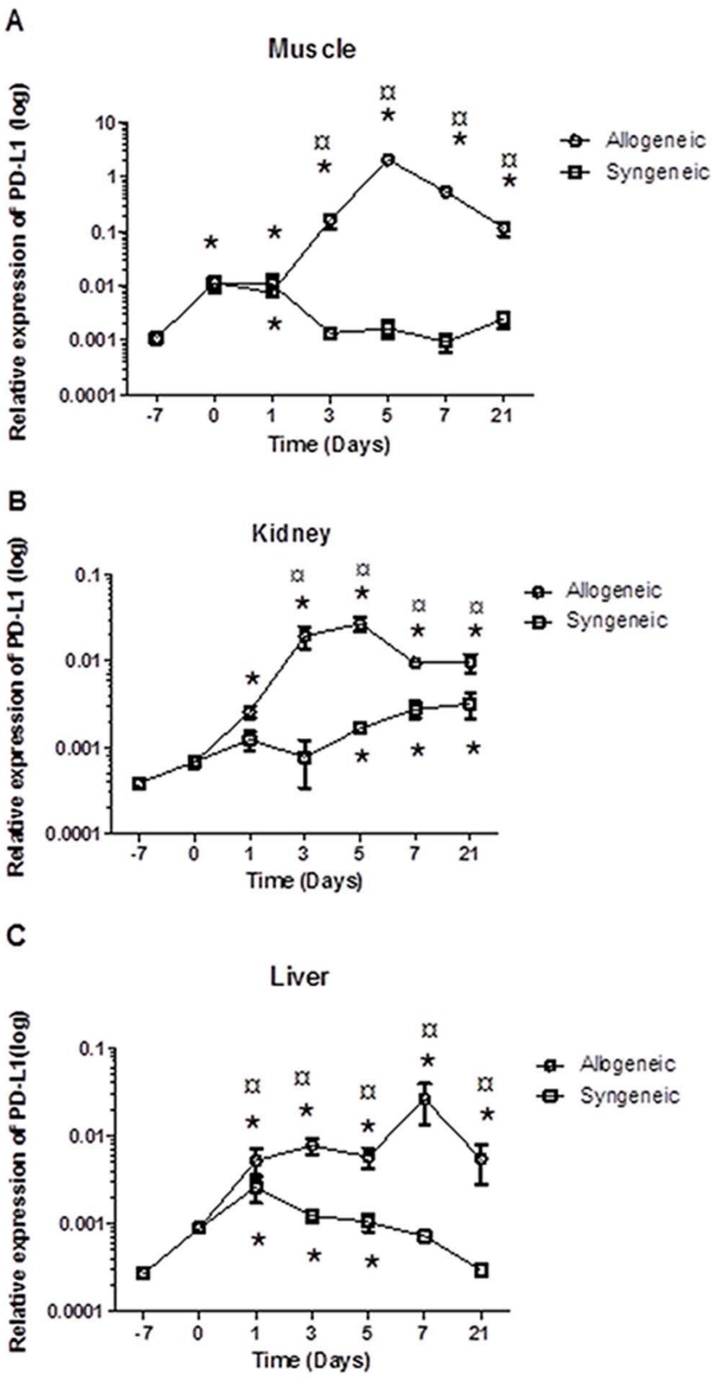

Figure 7. Dynamic expression of CD274 mRNA before and after bone marrow transplantation (syngeneic and allogeneic). Samples from control mice (D-7, before treatment), conditioned mice (D0) and allogeneic- and syngeneic-transplanted mice at different intervals (D+1, D+3, D+5, D+7 and D+21) from the muscle, kidney and liver were collected, mRNA was prepared and qPCR was performed. Normalized (PD-L1) expression was calculated relative to ACTB. The results are presented as mean ± SE.

a. Muscle b. Kidney c. Liver

Statistically different ($p<0.05$*) from the control value (D-7) as determined by the ANOVA test.

¤ Statistically different (*$p<0.05$*) from the syngeneic-transplanted mice as determined by the ANOVA test.

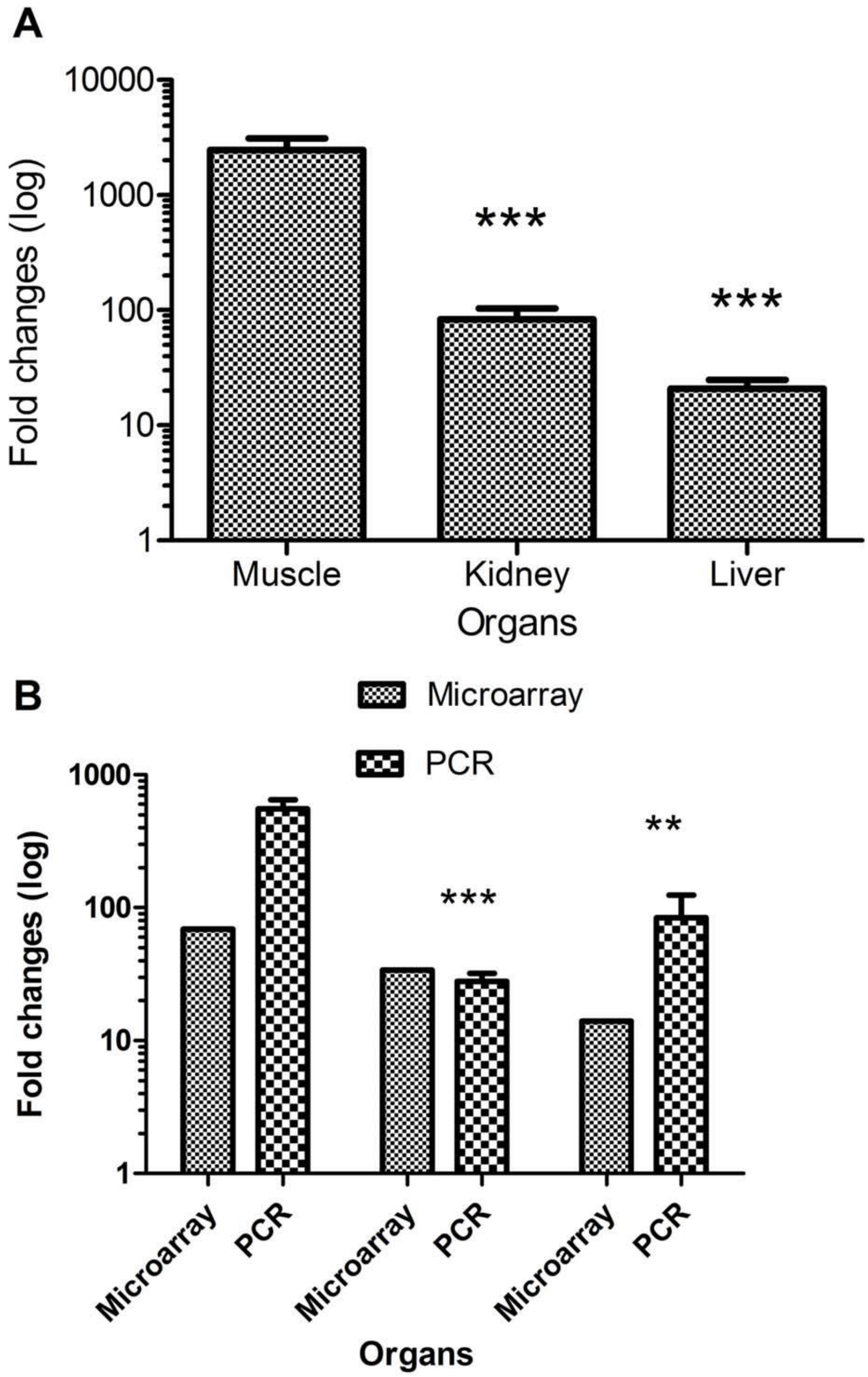

Figure 8. Comparison of mRNA expression fold changes**.** Fold changes of mRNA expression post allogeneic transplantation relative to control.

a. Fold changes (D+5) of mRNA for muscle and kidney and liver relative to controls (D-7).

b. Fold changes (D+7) post allogeneic transplantation, compared to microarray data of mRNA fold changes at the same day

** Significant (*p<0.01*)

*** Significant (*p<0.001*)

4.2.4 PD-L1 at the protein level

Western blot technique showed that mice receiving allogeneic transplants exhibited significantly increased levels of PD-L1 protein in all tested organs 5 days post transplantation. However, PD-L1 molecules in tissue samples from syngeneic-transplanted mice at any time point did not express PD-L1 (Figure 9).These increased levels were more pronounced in the muscle and kidney and remained at the same level even on day 7 post transplantation (Figure 9).

The immunohistochemical analysis also demonstrated that the expression of PD-L1 was up regulated in the muscle, kidney and liver of transplanted mice 5 days post allogeneic transplantation, and that the strongest up-regulation was observed in endothelial cells (Figure 10).

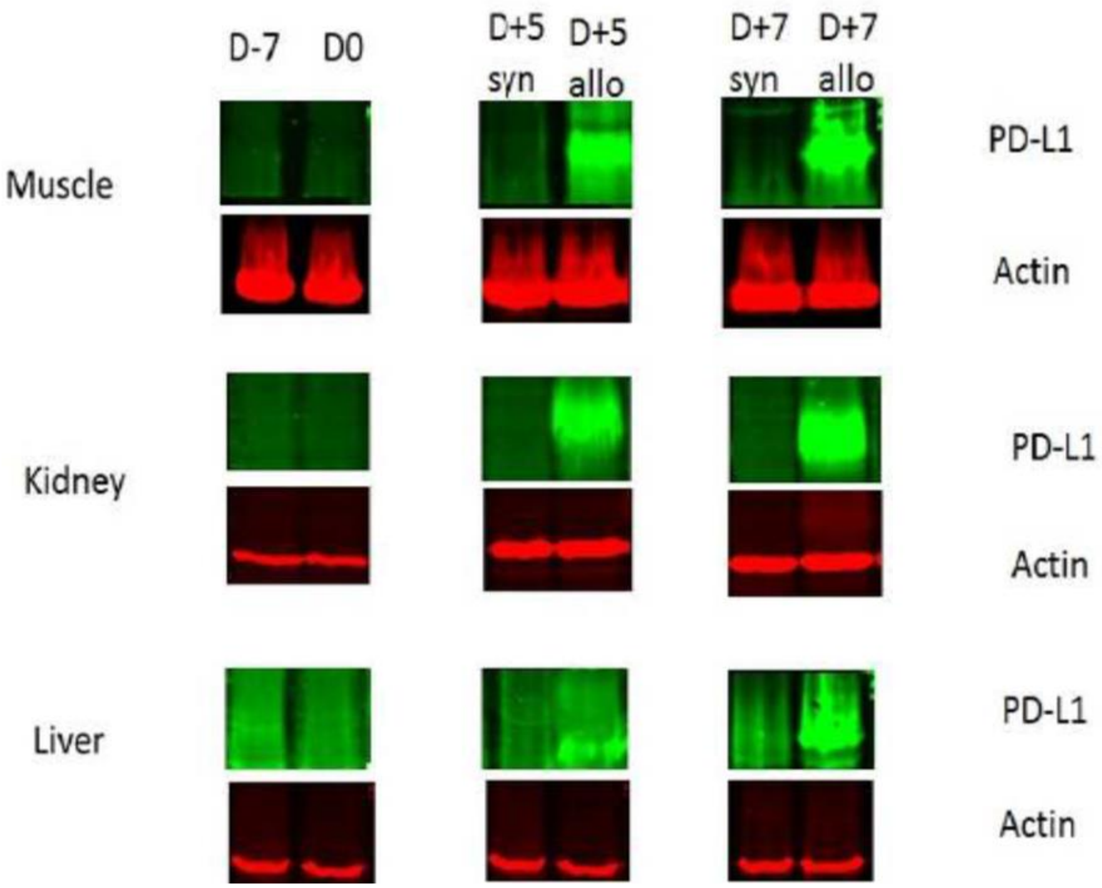

Figure 9. PD-L1 expression at the protein level as determined by western blot. Samples were taken from control mice (D-7), conditioned mice (D0) and allogeneic- and syngeneic-transplanted mice at different time points (D+5, D+7). Muscle, kidney and liver were collected, and the lysate was prepared and used for western blot.

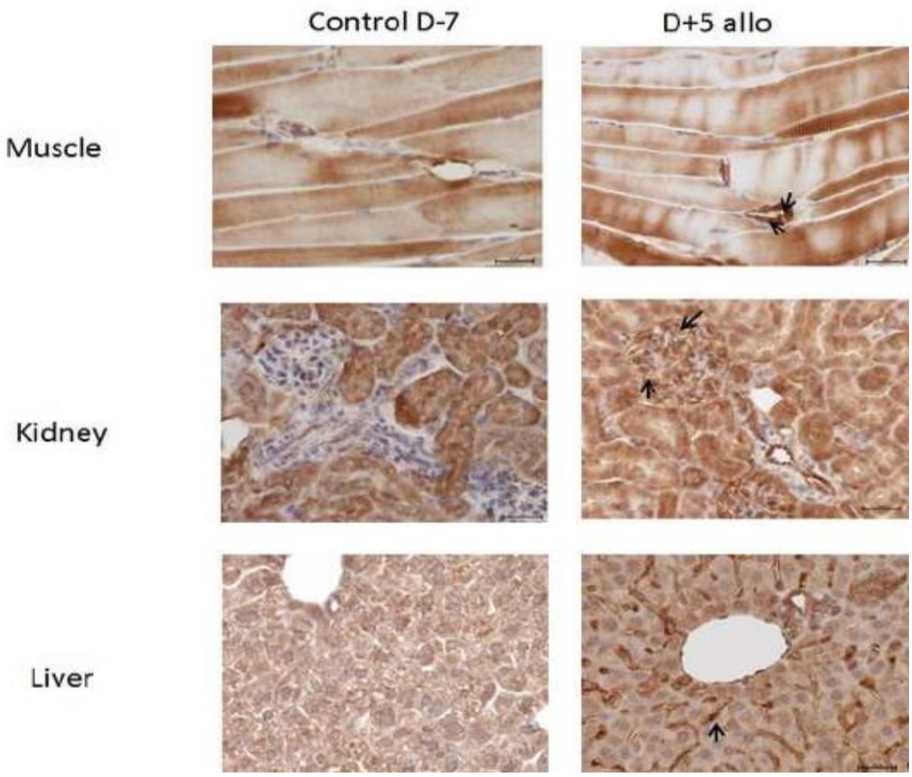

Figure 10. Expression of PD-L1 by immunohistochemistry**.** Immunohistochemistry staining for PD-L1 was positive (arrow) after allogeneic transplantation compared to controls (D-7) in all tissues. The strongest PD-L1 staining was obtained in the endothelial cells. Magnification 40X was used in all slides.

4.2.5 IFN-γ and TNF-α during acute GVHD development

It has been shown that inflammatory cytokines, IFN-γ and TNF-α in particular, play a crucial role in the development of GVHD.[196] These cytokines are also shown to be involved in the induction of PD-L1 expression.[197] Therefore, we evaluated the production of IFN-γ and TNF-α in mice subjected to syngeneic or allogeneic transplantation. As shown in Figures 11a and 11b, serum levels of these cytokines were significantly ($p<0.01$) enhanced in all treated animals after Bu-Cy chemotherapy (day0). Moreover, the enhancement of IFN-γ and TNF-α serum levels was further potentiated in allogeneic- but not syngeneic-transplanted mice, exhibiting a peak on day +5 and a slight decline on day 7 post transplantation.

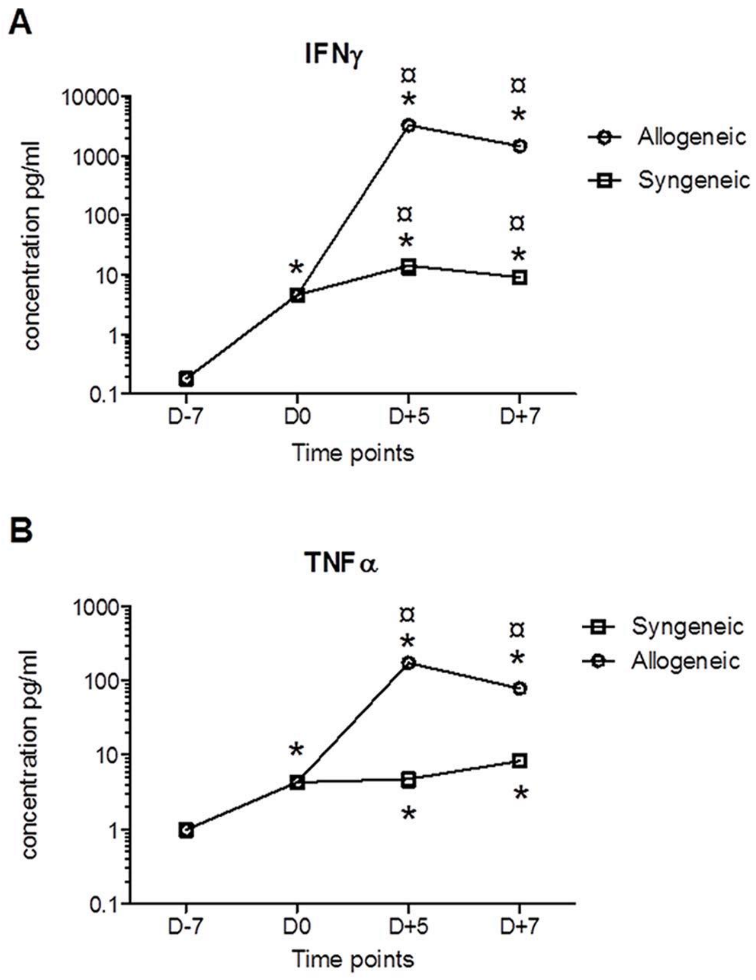

Figure 11. Kinetics of the production of inflammatory cytokines IFN-γ and TNF-α after chemotherapy based conditioning and transplantation with syngeneic or allogeneic bone marrow and splenic cells. Serum levels of (A) IFN-γ and (B) TNF-α**,** measured employing ELISA techniques.

*Statistically different (*p<0.05*) from the control value (D-7) as determined by the ANOVA test.

¤ Statistically different (*p<0.05*) from the syngeneic-transplanted mice as determined by the ANOVA test.

4.3 PAPER III

4.3.1 Gene expression after single-dose or fractionated TBI

Conditioning with fractionated TBI resulted in up- and down-regulation of a total 1065, 625 and 184 genes in the lung, muscle and liver respectively, while treatment with single-dose TBI specifically altered the expression of a total 3960, 30 and 17 genes in the same respective organs (Figure 12).

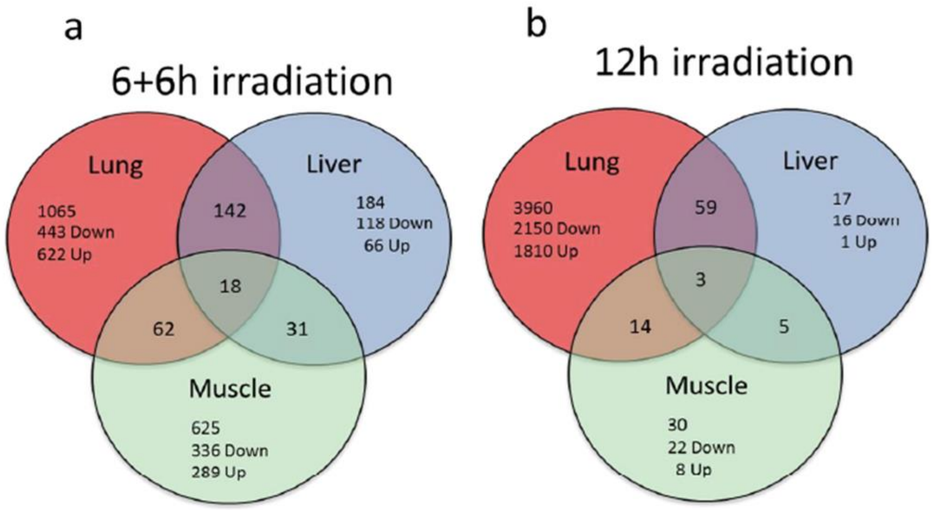

Figure 12. Effect of fractionated or single-dose total body irradiation (TBI) on the gene expression profile in the lung, liver and muscle. (a)Fractionated (b) Single-dose TBI after 12h.

Although, both conditioning regimens affected the lung predominantly, these regimens induce different patterns of gene expression in this organ, i.e. out of 3960 up and/or down regulated genes by single-dose TBI, only 74 genes are shared with those induced by the fractionated TBI (Figure 13).

In the lung, fractionated TBI predominantly up regulated the genes related to inflammation and innate immunity (e.g. inflammatory cytokine production, Toll-like, interferon and interleukin receptor signaling). Furthermore, the magnitude of these up-regulated genes in the lung did not exceed more than 4-fold. In contrast, treatment with single-dose TBI largely down regulated the genes related to antigen presentation, inflammatory responses and innate immunity (e.g. inflammatory cytokine production, chemokines, interferon, and interleukin receptor signaling). However, this conditional regimen significantly up regulated the genes relating to the activation of natural killer (NK) cells.

Together, these observations clearly imply that the lung is highly susceptible to both single-dose and fractionated TBI conditioning regimens. Moreover, single-dose TBI predominantly down regulated the genes related to immune and inflammatory response in the lung and this might, at least in part, explain why this conditioning regimen is more potent than fractionated TBI in induction of idiopathic interstitial pneumonia.[198] In addition, our observation that the genes of inflammatory responses were up regulated in the lung after administration of fractionated TBI implies that this regimen might be more involved than the single-dose TBI in induction and development of pulmonary GVHD, a complication that requires inflammatory cytokines.[199]

4.3.2 Kinetics of gene expression after single-dose TBI

Evaluation of the kinetics of gene expression in the lung as well as the liver and muscle upon treatment with single-dose TBI showed that the total numbers of up- and down regulated genes were different in each organ (Figure14 a-c) at different time-points (6, 12 and 72 hours). However, at each time-point, the lungs had the highest number of altered genes as compared to the liver and muscle (Figure 14a-c).

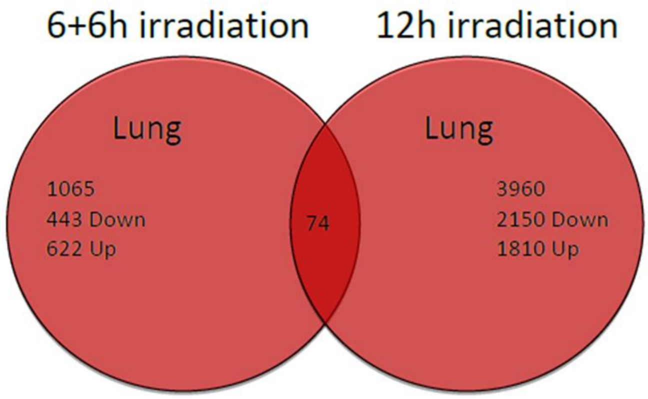

Figure 13. Fractionated or single-dose total body irradiation (TBI) differentially affect the gene expression in the lung.

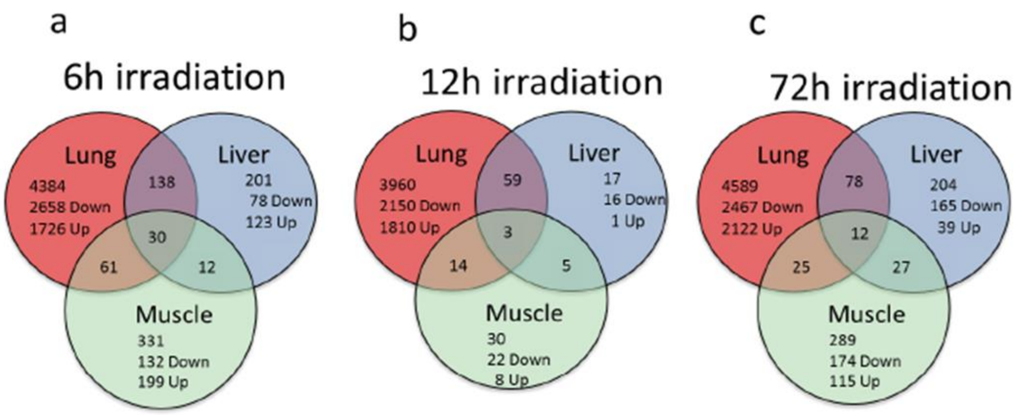

Figure 14. Kinetics of gene expression profiles in the lung, liver and muscle of mice conditioned with single-dose TBI after (a) 6h, (b) 12h and (c) 72h.

Since the majority of immune-related genes were down regulated in the lung at 12h, we analyzed the alterations in these genes 6 and 72 hours after treatment with single-dose TBI. At 6h, this organ exhibited alterations in 109 immune-related genes of which only 4 were up regulated. The down regulating effects were intensive and included the genes related to inflammation (e.g. chemokines, interferon, interleukin, toll-like and TNF receptor pathways), NK cell activation and antigen presentation pathway.

Analysis of immune-related gene expression profile in the lungs 72 hours after treatment with single-dose TBI revealed that among 70 altered genes, 14 and 56 genes were up and down regulated, respectively. Again, the majority of down regulated genes were related to antigen presentation and inflammatory responses, in particular chemokines, interleukin, Toll-like and TNF receptor pathways and complement systems.

On the other hand, few genes related to the chemokines, interleukin and toll-like receptor pathways and antigen presentation were among up regulated genes.

In contrast to the lungs, single-dose or fractionated TBI induced alterations in few (3-10) immune-related genes in the liver and muscle at any time point. In the liver, these alterations included both up- and down-regulations in the genes related to inflammatory responses, whereas in the muscle the altered genes belonged to inflammation as well as antigen presentation.

4.4 PAPER IV

4.4.1 Gene expression profile in CKD derived monocytes

A two way unsupervised hierarchical clustering showed that 600 genes were up regulated, and 272 down regulated, in CKD patients compared to healthy controls with $P < 0.05$ and fold changes of 1.5 and more (Figure 15).

4.4.2 Pathway of up regulated genes in CKD patients

Pathway analysis showed different pathways related to inflammation were up regulated. Wnt signaling pathway, inflammation mediated by chemokines and cytokine signaling, TGF-β signaling pathway, integrin signaling pathway and interleukin signaling pathway were the most highly up regulated in CKD patients (Table 1).

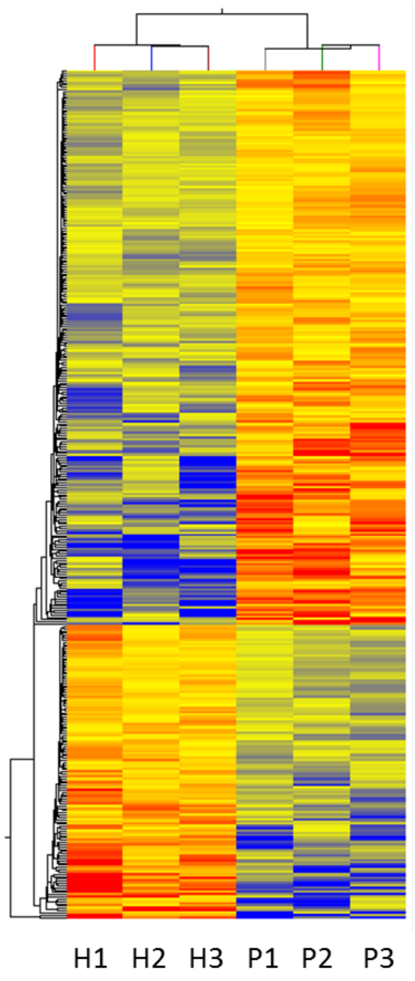

Figure 15. Gene expression pattern in peripheral monocytes from three healthy subjects (H1, H2, H3) and three patients with CKD (P1, P2, P3)

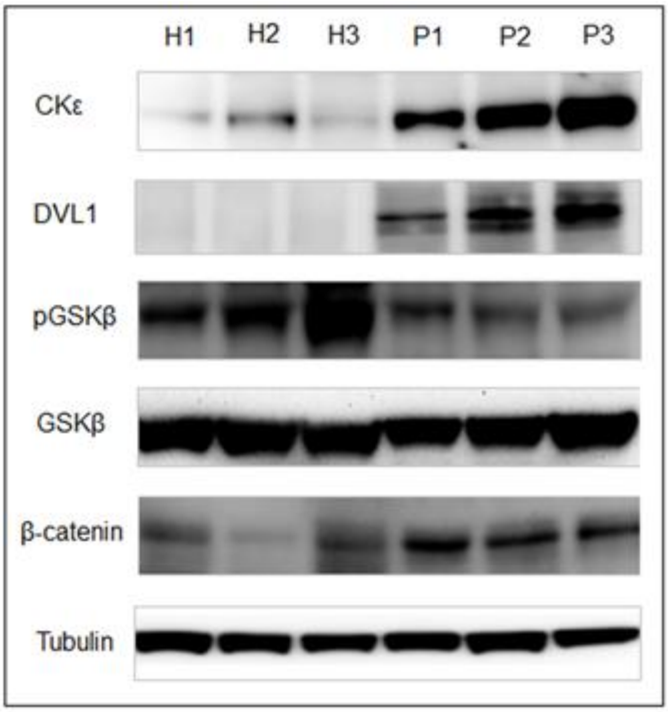

Figure 16. Representative Western blot of CK1ε, DVL1, PGSKβ, GSKβ, β-catenin, and Tubulin from three healthy subjects and three CKD patients

4.4.3 Wnt-β catenin pathway is activated in CKD monocytes

Different genes involved in the Wnt signaling pathway were up regulated in microarray gene expression results (Table 2). Follow up experiments by Western blot confirmed the presence of proteins involved in this pathway (Figure 16). Normally, in the inactive state β-catenin exists within a protein complex consisting of Axin, APC (adenomatous polyposis coli), GSKβ (Glycogen synthase kinase), and CK1ε (Casein Kinase) which leads to phosphorylation and destruction of β-catenin and prevents its accumulation and passing to the nucleus.[200] Activation of the canonical pathway ensues when Wnt proteins interact with specific cell surface receptor complexes that consist of members of the Frizzled family and low-density lipid receptor (LRP)-5 or LRP6. This triggers phosphorylation of disheveled proteins (DVL) and promotes their interaction with the frizzled proteins.[201, 202] The DVL/receptor complexes facilitate phosphorylation of the LRP6 intracellular tails by the CK1ε. As a consequence, Axin is recruited to this receptor complex and the degradation of β-catenin by proteasome is blocked. This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the TCF/LEF family and converts them into potent transcriptional activators.[203-206]

Table 1. Pathways up regulated in monocytes collected from patients with CKD stages 4 and 5

Name of pathway	No. of genes
Wnt signaling pathway	15
Inflammation mediated by chemokine and cytokine signaling pathway	12
TGF β signaling pathway	7
Integrin signaling pathway	6
interleukin signaling pathway	5

Table 2. Genes involved in Wnt signaling pathway

Mapped IDs	Fold change	Gene name
ANKRD6	1.6	Ankyrin repeat domain-containing protein 6
CDH15	1.7	Cadherin-15
CDH19	4.5	Cadherin-19
CDH8	3.2	Cadherin-8
DKK2	5	Dickkopf-related protein 2
EDN1	2.1	Big endothelin-1
FZD4	1.8	Frizzled-4
MYH8	1.9	Myosin-8
NFATC4	1.9	Nuclear factor of activated T-cells, cytoplasmic 4
PCDHA5	5.9	Protocadherin alpha-5
PCDHA9	2	Protocadherin alpha-9
PPARD	3.8	Peroxisome proliferator-activated receptor delta
PPP2R5C	1.9	Serine/threonine-protein phosphatase 2 regulatory subunit gamma isoform
TCF3	2.2	Transcription factor 7-like 1
WNT5A	5.6	Protein Wnt-5a

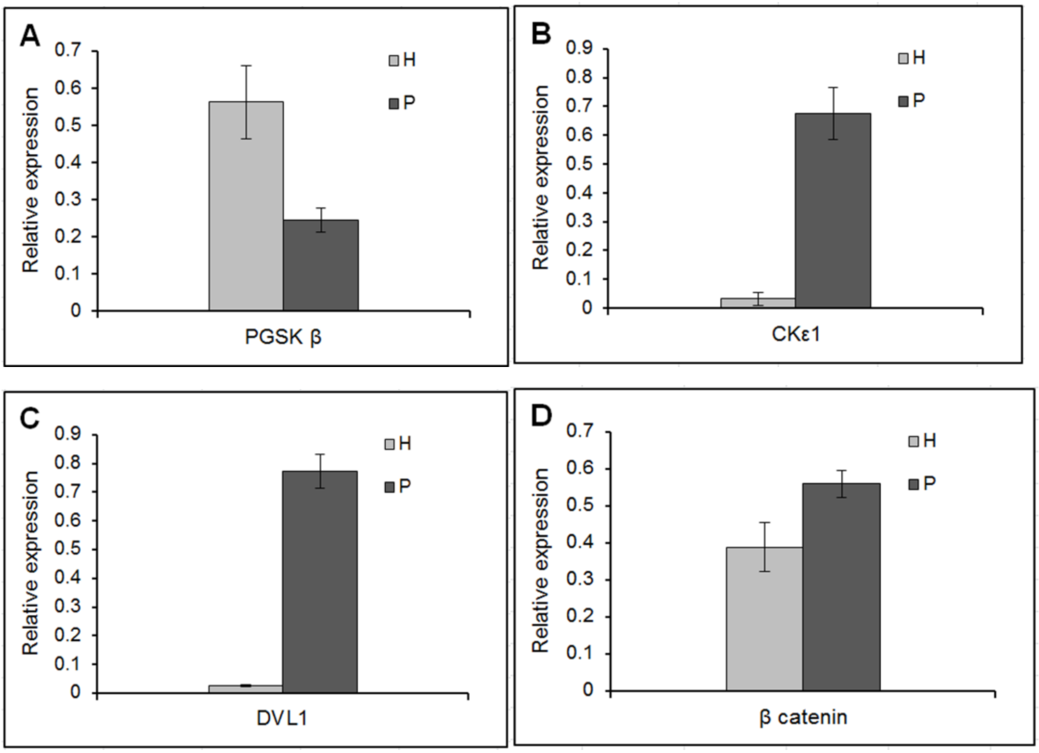

Figure 17. Densitometry of western blot from fourteen patients and ten healthy subjects. Tubulin was used to normalize the results of β-catenin, DVL1 and CK1ε while GSKβ was used to normalize PGSKβ.

A) PGSK ($p < 0.01$), B) CK1ε ($p < 0.0001$), C) DVL1 ($p < 0.00001$) and D) β-catenin ($p < 0.05$)

Both of the Figures (16) and (17a-d) show the Western blot and densitometry for quantification of β-catenin, GSKβ, PGSKβ, DVL1 and CK1ε respectively. The protein concentration is significantly different between the patients and the healthy group. β-catenin, which is the main protein in this pathway, was significantly more expressed in the patient group ($P < 0.05$). There was no difference in total GSKβ between the healthy and the patient groups, but the phosphorylated GSKβ (P GSKβ) was significantly more expressed in healthy subjects than in patients ($P < 0.01$). DVL1 and CK1ε were significantly more expressed in patients compared to in healthy subjects ($P < 0.00001$ and $P < 0.0001$, respectively).

5 DISCUSSION

Using microarray technique for global gene expression analysis provides a picture of all the transcriptional activity in a biological sample [207, 208] and thus facilitates the discovery of the changes in expression in a huge number of genes at the same time. It has been used widely to discover the marker of diseases and to understand more about the pathogenesis of the disease. In this thesis, the involvement of the immune system in pathogenesis of different diseases has been discussed thoroughly. Microarray gene expression was initially used as the main method to discover what is taking place.

HSCT has been used widely as the treatment of choice for several different malignant and nonmalignant conditions. Despite that, this type of treatment is accompanied by multiple side effects that hinder its use. Preparation for this treatment (i.e. conditioning) involves high risk in itself. Moreover, development of major complications, mainly GVHD, is a big obstacle in the way of successful HSCT. Different reports have shown that tissue damage due to conditioning and the following cytokine storm to be an important factor in initiating and developing GVHD [81, 163, 170, 209]

In our first study, gene expression profiles in different organs in the early stages of acute GVHD were studied. Organs differ in their liability to be affected by GVHD; some organs like liver and skin are more vulnerable.[210] Therefore, we wanted to expose the molecular changes in different organs after conditioning and HSCT and the development of acute GVHD. The finding that expression of CXCL1, ICAM1 and STAT3 along with the JAK–STAT pathway was significantly higher in the liver after chemotherapy conditioning suggested local inflammation and/or toxicity. The over expression of STAT3 in the liver, which is a mediator of the acute phase of the inflammatory response, could be caused by local tissue damage and inflammation observed in liver histology after conditioning.

An interesting finding was that during GVHD, progressive over expression of inflammatory genes was found in different organs in almost all tissues in allogeneic setting. This observation indicates the importance of allogeneicity and the role of donor cells in gene expression profile reflecting systemic inflammation at day +7.[211, 212] However, the differences in expression levels of these genes and/or relevant pathways in the liver and kidney of GVHD mice were significantly higher than those in the muscle of GVHD mice. Although 90% of biological processes and pathways in the liver and kidney of GVHD mice were related to inflammatory processes, only 25 to 30% of up regulated processes in the muscle belonged to these clusters.

Histopathological staining of the kidney of GVHD mice confirmed the presence of T cells (similar to that observed in the liver) indicating T-cell invasion to this organ after developing GVHD. Together these data indicate that the gene expression profile of the kidney in allo-SCT is comparable to that of the liver. However, expression patterns of inflammatory processes and immune response pathways in the kidney are different from those in the liver. It seems that these two organs experience different pathways provoking inflammation and immune response after allo-SCT and initiation of the GVHD process.

The impact of T cells on GVHD is well known, but the development of inhibiting factors or suppressing factors to the work of these T cells during the process of GVHD development is also important to consider. Therefore, we proceeded further in the second study to find out the kinetic of expression of PD-L1 in different organs known to be affected by GVHD and those known to be less affected by GVHD. In the past few years, different studies have focused on exploring the role of CD274 during HSCT.[213-215] However, results from microarrays in the first study revealed PD-L1/CD274 as the most highly expressed gene in the muscle on the seventh day after allogeneic transplantation.

PD-L1 is known for its inhibitory effect on activated T cells during its interaction with the PD-1 receptor on the surface of these cells.[216, 217] Expression of PD-L1 mRNA in the skeletal muscle of untreated control mice (D-7) was higher than that in the liver and kidney, and this strongly suggests that skeletal muscle constitutively expresses more PD-L1. The increase in mRNA levels of PD-L1 in all tested organs upon conditioning with Bu-Cy implies the possibility that conditioning-induced cytokine storm is responsible for the up-regulation of PD-L1 in different organs. In fact, this possibility is supported by our finding that serum levels of IFN-γ and TNF-α are significantly increased by conditioning prior to syngeneic or allogeneic transplantation.

The finding that PD-L1/CD274 was more expressed in the muscle, which is usually regarded as non- target organ, in acute GVHD supports the idea that PD-L1 may play an important role in protection of organs against developing GVHD. Moreover, its expression was more in the kidney than in the liver and this further strengthens the idea that it may protect organs during GVHD, or at least delay it. However, a question of chronicity arises, since it has been found that prolonged PD-L1 expression causes exhaustion of T cells and leads to the development of chronic inflammation.[218, 219]

Conditioning has been known to be associated with cytokine storm. However, conditioning is not a specific therapy; nearly all organs are affected by its toxic effects.

Optimization of the conditioning regimen is a critical point in reducing treatment related toxicity. Several reports addressed the effect of TBI given as single dose or in fractionated form, alone or in combination with cyclophosphamide. However, thorough investigations of the immunological effects of different strategies for providing TBI conditioning to different organs were missing. Therefore, in the third study the immunological effects of different strategies to provide TBI, single dose or fractionated, to different organs were investigated. The observation that single-dose (but not fractionated) TBI suppresses the expression of several genes related to both the innate and adaptive immune system suggests the tendency to develop interstitial pneumonitis after this type of conditioning. In particular, this applies to the major histocompatibility complex type II-associated invariant chain CD274, which is highly expressed by type II alveolar epithelial cells,[220]. Since histocompatibility importance in the pathogenesis of GVHD is well known, the observation that histocompatibility genes were strongly down regulated after single dose TBI further strengthens the idea that the incidence of pulmonary GVHD is lesser after single dose TBI. There is also higher risk of developing interstitial pneumonitis than pulmonary GVHD. In addition, our observation that the genes of inflammatory responses were up regulated in the lung after administration with fractionated TBI implies that this regimen might be more involved than the single-dose TBI in induction and development of pulmonary GVHD, a complication that requires inflammatory cytokines.[199]

However, the low magnitude of the changes in the gene expression after fractionated TBI infers that in general the splitting of dose induces less effect on tissues even when the total dose is similar to that administered in single dose TBI.

It is clear that the changes in the expression of genes leading further to production of different proteins on immune related cells is an issue worth studying during a course of immunological related disorder. Therefore, during our fourth project we attempted to find the gene expression changes in monocytes, which are critical cells in the immune system. We then studied these changes in monocytes from peripheral blood from chronic kidney disease patients, who are known for their low immunity and hence high liability to get infections.[177] Moreover, this patient group is characterized by a high mortality rate from cardiovascular complications, especially atherosclerosis.[221-223] Since monocytes are critical in the pathogenesis of atherosclerosis, [16, 224] the study of changes in gene expression in monocytes from CKD patients is important. Surprisingly, the gene expression showed a distinct difference between patients and the healthy group. Genes involved in pathways related to immune response and cell adhesion were up regulated. Furthermore, an important signaling pathway, the Wnt/β-catenin pathway, was significantly different in both groups. Activation of this pathway leads to accumulation of β-catenin in the cytoplasm, which has two impacts. First it will pass to the nucleus and help in activation of transcription factor; secondly this β-catenin by itself increases the adherence abilities of the monocytes to the endothelium.[225, 226] Moreover, Wnt5a has been associated with increased IL6 production in the macrophages.[227] Since this group of patients suffers from low grade inflammation [188] we concluded that this pathway is critical in the dysfunction of monocytes in CKD, and that interference with this pathway may improve quality of life in these patients regarding immunity and CVS complications.

6 CONCLUSIONS

The studies involved in this thesis have shown:

- The inflammatory response that occurs during early acute GVHD is a general response that occurs even in organs that are not targets for acute GVHD, but to a lesser extent.

- Other organs than the usual known target organs for acute GVHD such as the kidney could be silent target organs for acute GVHD or may be late affected organs.

- PD-L1 could have a protective role against acute GVHD, may delay its development or may make it latent.

- Single dose and fractionated TBI produce different immunological responses in different organs.

- Wnt/β-catenin signaling is activated in monocytes from CKD patients. Interference with this pathway may improve immune function and reduce cardiovascular complications in this patient group.

7 ACKNOWLEDGEMENTS

I would like to thank all the people that supported me through the years of work on this study, especially my main supervisor Professor Moustapha Hassan who gave me the chance to do my PhD in Sweden. My co-supervisor Professor Joachim Lundahl for his enthusiasm, his support and the comfortable place of work he provided me in his group. Dr. Ali Moshfegh, my previous main supervisor, for introducing me to the gene expression world. Dr.Alan Fotoohi my mentor for worthy advices.

I want also to thank all my co-authors: Behnam Sadeghi, Sulaiman Al-Hashmi, Mona Fares, Josefin Paulsson, Professor Stefan Jacobson, Manuchehr Abedi-Valugerdi and Raoul Kuiper for their work with me in conducting these studies.

I would like to thank all the people in the LabMed department for their kindness when I was working in Novum, especially Jennifer Usterud.

Therese Jacobson, my first Swedish friend and roommate, thanks for your help in the lab and whenever I need it. Zenib Al-Jadi, Ladan Mansouri and all the people in L2:04 for the enjoyable time I spent among you.

I would like to thank Kurdistan regional government and ministry of health for their financial support. My Kurdish friends and people from KOMAR for their support.

Thank you to all my relatives and friends in Sweden and in Kurdistan for being helpful and supportive all the time.

My appreciation and thanks to my parents and parents-in-law for the prayer and endless love. You mean a lot to me.

Finally, I would like to say some words to my family. My children Ahmed, Abdullah and Dalya, thanks to God you were with me all the days. You are the most important thing in my life. I think you had a good experience here in Sweden with me.

At the end I would like to say a “thank you” that comes from the inside of my heart to the person who believed in me and was the reason I came to Sweden: my husband “Mohammed”.

8 REFERENCES

1. Dempsey, P.W., S.A. Vaidya, and G. Cheng, *The art of war: Innate and adaptive immune responses.* Cell Mol Life Sci, 2003. **60**(12): p. 2604-21.

2. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.

3. Moser, M. and O. Leo, *Key concepts in immunology.* Vaccine, 2010. **28 Suppl 3**: p. C2-13.

4. Janeway, C.A., Jr., et al., *CD4+ T cells: specificity and function.* Immunol Rev, 1988. **101**: p. 39-80.

5. Haas, W., P. Pereira, and S. Tonegawa, *Gamma/delta cells.* Annu Rev Immunol, 1993. **11**: p. 637-85.

6. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. 1986.* J Immunol, 2005. **175**(1): p. 5-14.

7. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annu Rev Immunol, 1989. **7**: p. 145-73.

8. Berke, G., *The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects.* Annu Rev Immunol, 1994. **12**: p. 735-73.

9. Auffray, C., M.H. Sieweke, and F. Geissmann, *Blood monocytes: development, heterogeneity, and relationship with dendritic cells.* Annu Rev Immunol, 2009. **27**: p. 669-92.

10. van Furth, R. and Z.A. Cohn, *The origin and kinetics of mononuclear phagocytes.* J Exp Med, 1968. **128**(3): p. 415-35.

11. van Furth, R., *Human monocytes and cytokines.* Res Immunol, 1998. **149**(7-8): p. 719-20.

12. Delneste, Y., et al., *Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages.* Blood, 2003. **101**(1): p. 143-50.

13. Worthylake, R.A. and K. Burridge, *Leukocyte transendothelial migration: orchestrating the underlying molecular machinery.* Curr Opin Cell Biol, 2001. **13**(5): p. 569-77.

14. Leon, B., M. Lopez-Bravo, and C. Ardavin, *Monocyte-derived dendritic cells.* Semin Immunol, 2005. **17**(4): p. 313-8.

15. Shi, C. and E.G. Pamer, *Monocyte recruitment during infection and inflammation.* Nat Rev Immunol, 2011. **11**(11): p. 762-74.

16. Woollard, K.J. and F. Geissmann, *Monocytes in atherosclerosis: subsets and functions.* Nat Rev Cardiol, 2010. **7**(2): p. 77-86.

17. Vyas, J.M., A.G. Van der Veen, and H.L. Ploegh, *The known unknowns of antigen processing and presentation.* Nat Rev Immunol, 2008. **8**(8): p. 607-18.

18. Greenwald, R.J., G.J. Freeman, and A.H. Sharpe, *The B7 family revisited.* Annu Rev Immunol, 2005. **23**: p. 515-48.

19. Seder, R.A., et al., *CD28-mediated costimulation of interleukin 2 (IL-2) production plays a critical role in T cell priming for IL-4 and interferon gamma production.* J Exp Med, 1994. **179**(1): p. 299-304.

20. Fife, B.T. and J.A. Bluestone, *Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways.* Immunol Rev, 2008. **224**: p. 166-82.

21. So, T., S.W. Lee, and M. Croft, *Tumor necrosis factor/tumor necrosis factor receptor family members that positively regulate immunity.* Int J Hematol, 2006. **83**(1): p. 1-11.

22. Copelan, E.A., *Hematopoietic stem-cell transplantation.* N Engl J Med, 2006. **354**(17): p. 1813-26.

23. Osawa, M., et al., *Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell.* Science, 1996. **273**(5272): p. 242-5.

24. Jackson, K.A., et al., *Stem cells: a minireview.* J Cell Biochem Suppl, 2002. **38**: p. 1-6.

25. Dreger, P., et al., *Indications for allogeneic stem cell transplantation in chronic lymphocytic leukemia: the EBMT transplant consensus.* Leukemia, 2007. **21**(1): p. 12-7.

26. Gratwohl, A., et al., *Hematopoietic stem cell transplantation for hematological malignancies in Europe.* Leukemia, 2003. **17**(5): p. 941-59.

27. Richardson, P. and E. Guinan, *Hepatic veno-occlusive disease following hematopoietic stem cell transplantation.* Acta Haematol, 2001. **106**(1-2): p. 57-68.

28. Ljungman, P., et al., *Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe 2009.* Bone Marrow Transplant, 2010. **45**(2): p. 219-34.

29. Gratwohl, A. and D. Niederwieser, *History of hematopoietic stem cell transplantation: evolution and perspectives.* Curr Probl Dermatol, 2012. **43**: p. 81-90.

30. de la Morena, M.T. and R.A. Gatti, *A history of bone marrow transplantation.* Hematol Oncol Clin North Am, 2011. **25**(1): p. 1-15.

31. Appelbaum, F.R., *The current status of hematopoietic cell transplantation.* Annu Rev Med, 2003. **54**: p. 491-512.

32. Meadows, M., *Bone marrow transplants come of age. New hope for deadly diseases.* FDA Consum, 2000. **34**(4): p. 22-7.

33. Jacobson, L.O., E.K. Marks, and E.O. Gaston, *[Effect of protection of the spleen during total body irradiation on the blood in rabbit]*. Rev Hematol, 1953. **8**(4): p. 515-32.

34. Lorenz, E., et al., *Modification of irradiation injury in mice and guinea pigs by bone marrow injections.* J Natl Cancer Inst, 1951. **12**(1): p. 197-201.

35. Thomas, E.D., et al., *Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy.* N Engl J Med, 1957. **257**(11): p. 491-6.

36. Storb, R., et al., *Marrow grafts by combined marrow and leukocyte infusions in unrelated dogs selected by histocompatibility typing.* Transplantation, 1968. **6**(4): p. 587-93.

37. Epstein, R.B., et al., *Autologous bone marrow grafts in dogs treated with lethal doses of cyclophosphamide.* Cancer Res, 1969. **29**(5): p. 1072-5.

38. Storb, R., et al., *Allogeneic canine bone marrow transplantation following cyclophosphamide.* Transplantation, 1969. **7**(5): p. 378-86.

39. Walasek, M.A., R. van Os, and G. de Haan, *Hematopoietic stem cell expansion: challenges and opportunities.* Ann N Y Acad Sci, 2012. **1266**: p. 138-50.

40. Ringden, O. and K. Le Blanc, *Allogeneic hematopoietic stem cell transplantation: state of the art and new perspectives.* APMIS, 2005. **113**(11-12): p. 813-30.

41. Ringden, O., et al., *Transplantation of peripheral blood stem cells as compared with bone marrow from HLA-identical siblings in adult patients with acute myeloid leukemia and acute lymphoblastic leukemia.* J Clin Oncol, 2002. **20**(24): p. 4655-64.

42. Bensinger, W.I., et al., *Transplantation of bone marrow as compared with peripheral-blood cells from HLA-identical relatives in patients with hematologic cancers.* N Engl J Med, 2001. **344**(3): p. 175-81.

43. Mohty, M., et al., *Higher doses of CD34+ peripheral blood stem cells are associated with increased mortality from chronic graft-versus-host disease after allogeneic HLA-identical sibling transplantation.* Leukemia, 2003. **17**(5): p. 869-75.

44. Cutler, C., et al., *Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: a meta-analysis.* J Clin Oncol, 2001. **19**(16): p. 3685-91.

45. Wagner, J.E. and J. Kurtzberg, *Cord blood stem cells.* Curr Opin Hematol, 1997. **4**(6): p. 413-8.

46. Cohena, Y. and A. Nagler, *Hematopoietic stem-cell transplantation using umbilical-cord blood.* Leuk Lymphoma, 2003. **44**(8): p. 1287-99.

47. Barker, J.N., *Umbilical Cord Blood (UCB) transplantation: an alternative to the use of unrelated volunteer donors?* Hematology Am Soc Hematol Educ Program, 2007: p. 55-61.

48. Horton, R., et al., *Gene map of the extended human MHC.* Nat Rev Genet, 2004. **5**(12): p. 889-99.

49. Trowsdale, J., *The MHC, disease and selection.* Immunol Lett, 2011. **137**(1-2): p. 1-8.

50. Petersdorf, E.W. and M. Malkki, *Genetics of risk factors for graft-versus-host disease.* Semin Hematol, 2006. **43**(1): p. 11-23.

51. Woolfrey, A., et al., *HLA-C antigen mismatch is associated with worse outcome in unrelated donor peripheral blood stem cell transplantation.* Biol Blood Marrow Transplant, 2011. **17**(6): p. 885-92.

52. Beatty, P.G., et al., *Marrow transplantation from related donors other than HLA-identical siblings.* N Engl J Med, 1985. **313**(13): p. 765-71.

53. Anasetti, C., et al., *Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma.* Hum Immunol, 1990. **29**(2): p. 79-91.

54. Davies, S.M., et al., *Unrelated donor bone marrow transplantation: influence of HLA A and B incompatibility on outcome.* Blood, 1995. **86**(4): p. 1636-42.

55. Eapen, M., et al., *Effect of donor-recipient HLA matching at HLA A, B, C, and DRB1 on outcomes after umbilical-cord blood transplantation for leukaemia and myelodysplastic syndrome: a retrospective analysis.* Lancet Oncol, 2011. **12**(13): p. 1214-21.

56. Anasetti, C., et al., *Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma.* N Engl J Med, 1989. **320**(4): p. 197-204.

57. Nowak, J., *Role of HLA in hematopoietic SCT.* Bone Marrow Transplant, 2008. **42 Suppl 2**: p. S71-6.

58. Klingebiel, T., et al., *Haploidentical transplantation for acute lymphoblastic leukemia in childhood.* Blood Rev, 2004. **18**(3): p. 181-92.

59. Huang, X.J., *Current status of haploidentical stem cell transplantation for leukemia.* J Hematol Oncol, 2008. **1**: p. 27.

60. Sage, D., *My approach to the immunogenetics of haematopoietic stem cell transplant matching.* J Clin Pathol, 2010. **63**(3): p. 194-8.

61. Cheuk, D.K., et al., *Risk factors and mortality predictors of hepatic veno-occlusive disease after pediatric hematopoietic stem cell transplantation.* Bone Marrow Transplant, 2007. **40**(10): p. 935-44.

62. Bjorklund, A., et al., *Risk factors for fatal infectious complications developing late after allogeneic stem cell transplantation.* Bone Marrow Transplant, 2007. **40**(11): p. 1055-62.

63. Mickelson, E.M., E.W. Petersdorf, and J.A. Hansen, *HLA matching and hematopoietic cell transplant outcome.* Clin Transpl, 2002: p. 263-71.

64. Myers, G.D., et al., *Reconstitution of adenovirus-specific cell-mediated immunity in pediatric patients after hematopoietic stem cell transplantation.* Bone Marrow Transplant, 2007. **39**(11): p. 677-86.

65. Godder, K.T., et al., *Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation.* Bone Marrow Transplant, 2007. **39**(12): p. 751-7.

66. Ringden, O., et al., *Similar incidence of graft-versus-host disease using HLA-A, -B and -DR identical unrelated bone marrow donors as with HLA-identical siblings.* Bone Marrow Transplant, 1995. **15**(4): p. 619-25.

67. Horowitz, M.M., et al., *Graft-versus-leukemia reactions after bone marrow transplantation.* Blood, 1990. **75**(3): p. 555-62.

68. Xun, C.Q., et al., *Effect of total body irradiation, busulfan-cyclophosphamide, or cyclophosphamide conditioning on inflammatory cytokine release and development of acute and chronic graft-versus-host disease in H-2-incompatible transplanted SCID mice.* Blood, 1994. **83**(8): p. 2360-7.

69. Haraguchi, K., et al., *Host-residual invariant NK T cells attenuate graft-versus-host immunity.* J Immunol, 2005. **175**(2): p. 1320-8.

70. Shlomchik, W.D., et al., *Prevention of graft versus host disease by inactivation of host antigen-presenting cells.* Science, 1999. **285**(5426): p. 412-5.

71. Vriesendorp, H.M., *Aims of conditioning.* Exp Hematol, 2003. **31**(10): p. 844-54.

72. Copelan, E.A., *Conditioning regimens for allogeneic bone marrow transplantation.* Blood Rev, 1992. **6**(4): p. 234-42.

73. Cremer, B., et al., *Reduced-intensity conditioning in allogeneic stem cell transplantation for hematological malignancies: a historical perspective.* Onkologie, 2011. **34**(12): p. 710-5.

74. Forman, S.J., *What is the role of reduced-intensity transplantation in the treatment of older patients with AML?* Hematology Am Soc Hematol Educ Program, 2009: p. 406-13.

75. Kiss, T.L., et al., *Blood and marrow transplantation in elderly acute myeloid leukaemia patients - older certainly is not better.* Bone Marrow Transplant, 2007. **40**(5): p. 405-16.

76. Baron, F. and R. Storb, *Hematopoietic cell transplantation after reduced-intensity conditioning for older adults with acute myeloid leukemia in complete remission.* Curr Opin Hematol, 2007. **14**(2): p. 145-51.

77. Verneris, M.R., et al., *Reduced-intensity conditioning regimens for allogeneic transplantation in children with acute lymphoblastic leukemia.* Biol Blood Marrow Transplant, 2010. **16**(9): p. 1237-44.

78. Veys, P., K. Rao, and P. Amrolia, *Stem cell transplantation for congenital immunodeficiencies using reduced-intensity conditioning.* Bone Marrow Transplant, 2005. **35 Suppl 1**: p. S45-7.

79. Satwani, P., et al., *Reduced intensity conditioning and allogeneic stem cell transplantation in childhood malignant and nonmalignant diseases.* Bone Marrow Transplant, 2008. **41**(2): p. 173-82.

80. Ringden, O., et al., *Reduced intensity conditioning compared with myeloablative conditioning using unrelated donor transplants in patients with acute myeloid leukemia.* J Clin Oncol, 2009. **27**(27): p. 4570-7.

81. Perez-Simon, J.A., et al., *Influence of the intensity of the conditioning regimen on the characteristics of acute and chronic graft-versus-host disease after allogeneic transplantation.* Br J Haematol, 2005. **130**(3): p. 394-403.

82. Ozsahin, M., et al., *Morbidity After Total Body Irradiation.* Semin Radiat Oncol, 1994. **4**(2): p. 95-102.

83. Hill-Kayser, C.E., et al., *TBI during BM and SCT: review of the past, discussion of the present and consideration of future directions.* Bone Marrow Transplant, 2011. **46**(4): p. 475-84.

84. Thomas, E.D., et al., *Bone-marrow transplantation (second of two parts).* N Engl J Med, 1975. **292**(17): p. 895-902.

85. Altschuler, C., et al., *Fractionated total body irradiation and bone marrow transplantation in acute lymphoblastic leukemia.* Int J Radiat Oncol Biol Phys, 1990. **19**(5): p. 1151-4.

86. Keane, T.J., J. Van Dyk, and W.D. Rider, *Idiopathic interstitial pneumonia following bone marrow transplantation: the relationship with total body irradiation.* Int J Radiat Oncol Biol Phys, 1981. **7**(10): p. 1365-70.

87. Kim, T.H., et al., *Interstitial pneumonitis following total body irradiation for bone marrow transplantation using two different dose rates.* Int J Radiat Oncol Biol Phys, 1985. **11**(7): p. 1285-91.

88. Barrett, A.J., *Bone marrow transplantation.* Cancer Treat Rev, 1987. **14**(3-4): p. 203-13.

89. Barrett, A., M.H. Depledge, and R.L. Powles, *Interstitial pneumonitis following bone marrow transplantation after low dose rate total body irradiation.* Int J Radiat Oncol Biol Phys, 1983. **9**(7): p. 1029-33.

90. Thomas, E.D., et al., *Marrow transplantation for acute nonlymphoblastic leukemic in first remission using fractionated or single-dose irradiation.* Int J Radiat Oncol Biol Phys, 1982. **8**(5): p. 817-21.

91. Katz, R., S.C. Sharma, and M. Homayoonfar, *Irradiation equivalence.* Health Phys, 1972. **23**(5): p. 740-1.

92. Deeg, H.J. and R.P. Witherspoon, *Risk factors for the development of secondary malignancies after marrow transplantation.* Hematol Oncol Clin North Am, 1993. **7**(2): p. 417-29.

93. Deeg, H.J., et al., *Cataracts after total body irradiation and marrow transplantation: a sparing effect of dose fractionation.* Int J Radiat Oncol Biol Phys, 1984. **10**(7): p. 957-64.

94. Benyunes, M.C., et al., *Cataracts after bone marrow transplantation: long-term follow-up of adults treated with fractionated total body irradiation.* Int J Radiat Oncol Biol Phys, 1995. **32**(3): p. 661-70.

95. Giorgiani, G., et al., *Role of busulfan and total body irradiation on growth of prepubertal children receiving bone marrow transplantation and results of treatment with recombinant human growth hormone.* Blood, 1995. **86**(2): p. 825-31.

96. Thomas, E.D., et al., *One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation.* Blood, 1977. **49**(4): p. 511-33.

97. de Jonge, M.E., et al., *Clinical pharmacokinetics of cyclophosphamide.* Clin Pharmacokinet, 2005. **44**(11): p. 1135-64.

98. Colvin, O.M., *An overview of cyclophosphamide development and clinical applications.* Curr Pharm Des, 1999. **5**(8): p. 555-60.

99. Cohen, J.L. and J.Y. Jao, *Enzymatic basis of cyclophosphamide activation by hepatic microsomes of the rat.* J Pharmacol Exp Ther, 1970. **174**(2): p. 206-10.

100. Moore, M.J., *Clinical pharmacokinetics of cyclophosphamide.* Clin Pharmacokinet, 1991. **20**(3): p. 194-208.

101. Hassan, M. and B.S. Andersson, *Role of pharmacogenetics in busulfan/cyclophosphamide conditioning therapy prior to hematopoietic stem cell transplantation.* Pharmacogenomics, 2013. **14**(1): p. 75-87.

102. Fried, W., A. Kedo, and J. Barone, *Effects of cyclophosphamide and of busulfan on spleen colony-forming units and on hematopoietic stroma.* Cancer Res, 1977. **37**(4): p. 1205-9.

103. Galton, D.A., *Myleran in chronic myeloid leukaemia; results of treatment.* Lancet, 1953. **264**(6753): p. 208-13.

104. Santos, G.W., *The development of busulfan/cyclophosphamide preparative regimens.* Semin Oncol, 1993. **20**(4 Suppl 4): p. 12-6; quiz 17.

105. Santos, G.W., *Preparative regimens: chemotherapy versus chemoradiotherapy. A historical perspective.* Ann N Y Acad Sci, 1995. **770**: p. 1-7.

106. McCune, J.S. and L.A. Holmberg, *Busulfan in hematopoietic stem cell transplant setting.* Expert Opin Drug Metab Toxicol, 2009. **5**(8): p. 957-69.

107. Ciurea, S.O. and B.S. Andersson, *Busulfan in hematopoietic stem cell transplantation.* Biol Blood Marrow Transplant, 2009. **15**(5): p. 523-36.

108. Weatherall, D.J., D.A. Galton, and H.E. Kay, *Busulphan and bone marrow depression.* Br Med J, 1969. **1**(5644): p. 638.

109. Hassan, M. and H. Ehrsson, *Metabolism of 14C-busulfan in isolated perfused rat liver.* Eur J Drug Metab Pharmacokinet, 1987. **12**(1): p. 71-6.

110. DeLeve, L.D. and X. Wang, *Role of oxidative stress and glutathione in busulfan toxicity in cultured murine hepatocytes.* Pharmacology, 2000. **60**(3): p. 143-54.

111. Nilsson, C., et al., *Effect of altering administration order of busulphan and cyclophosphamide on the myeloablative and immunosuppressive properties of the conditioning regimen in mice.* Exp Hematol, 2005. **33**(3): p. 380-7.

112. Sadeghi, B., et al., *The effect of administration order of BU and CY on engraftment and toxicity in HSCT mouse model.* Bone Marrow Transplant, 2008. **41**(10): p. 895-904.

113. Boeckh, M. and P. Ljungman, *How we treat cytomegalovirus in hematopoietic cell transplant recipients.* Blood, 2009. **113**(23): p. 5711-9.

114. Einsele, H., et al., *Infectious complications after allogeneic stem cell transplantation: epidemiology and interventional therapy strategies--guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Hematology and Oncology (DGHO).* Ann Hematol, 2003. **82 Suppl 2**: p. S175-85.

115. Ninin, E., et al., *Longitudinal study of bacterial, viral, and fungal infections in adult recipients of bone marrow transplants.* Clin Infect Dis, 2001. **33**(1): p. 41-7.

116. Salutari, P., et al., *Incidence of sepsis after peripheral blood progenitor cells transplantation: analysis of 86 consecutive hemato oncological patients.* Leuk Lymphoma, 1998. **30**(1-2): p. 193-7.

117. Leather, H.L. and J.R. Wingard, *Infections following hematopoietic stem cell transplantation.* Infect Dis Clin North Am, 2001. **15**(2): p. 483-520.

118. Meyers, J.D. and K. Atkinson, *Infection in bone marrow transplantation.* Clin Haematol, 1983. **12**(3): p. 791-811.

119. Breuer, S., et al., *Molecular diagnosis and management of viral infections in hematopoietic stem cell transplant recipients.* Mol Diagn Ther, 2012. **16**(2): p. 63-77.

120. Tabbara, I.A., et al., *Allogeneic hematopoietic stem cell transplantation: complications and results.* Arch Intern Med, 2002. **162**(14): p. 1558-66.

121. Coppell, J.A., et al., *Hepatic veno-occlusive disease following stem cell transplantation: incidence, clinical course, and outcome.* Biol Blood Marrow Transplant, 2010. **16**(2): p. 157-68.

122. McDonald, G.B., et al., *Veno-occlusive disease of the liver and multiorgan failure after bone marrow transplantation: a cohort study of 355 patients.* Ann Intern Med, 1993. **118**(4): p. 255-67.

123. Champlin, R.E., et al., *Graft failure following bone marrow transplantation for severe aplastic anemia: risk factors and treatment results.* Blood, 1989. **73**(2): p. 606-13.

124. Jung, M.H., et al., *Endocrine complications after hematopoietic stem cell transplantation during childhood and adolescence.* J Korean Med Sci, 2009. **24**(6): p. 1071-7.

125. Lee, V., et al., *Autoimmune hypothyroidism after unrelated haematopoietic stem cell transplantation in children.* J Pediatr Hematol Oncol, 2006. **28**(5): p. 293-5.

126. Au, W.Y., et al., *Autoimmune thyroid dysfunction after hematopoietic stem cell transplantation.* Bone Marrow Transplant, 2005. **35**(4): p. 383-8.

127. Cohen, A., et al., *Endocrinological late complications after hematopoietic SCT in children.* Bone Marrow Transplant, 2008. **41 Suppl 2**: p. S43-8.

128. Roziakova, L. and B. Mladosievicova, *Endocrine late effects after hematopoietic stem cell transplantation.* Oncol Res, 2010. **18**(11-12): p. 607-15.

129. Kal, H.B. and V.A.N.K.-H. ML, *Induction of severe cataract and late renal dysfunction following total body irradiation: dose-effect relationships.* Anticancer Res, 2009. **29**(8): p. 3305-9.

130. Claes, K. and P. Kestelyn, *Ocular manifestations of graft versus host disease following bone marrow transplantation.* Bull Soc Belge Ophtalmol, 2000(277): p. 21-6.

131. Woodard, P., et al., *Brain parenchymal damage after haematopoietic stem cell transplantation for severe sickle cell disease.* Br J Haematol, 2005. **129**(4): p. 550-2.

132. Hilgendorf, I., et al., *Neurological complications after intrathecal liposomal cytarabine application in patients after allogeneic haematopoietic stem cell transplantation.* Ann Hematol, 2008. **87**(12): p. 1009-12.

133. Brey, R.L., *Severe neurologic complications after hematopoietic (peripheral blood) stem cell transplantation in children.* Neurology, 2002. **59**(12): p. E13-5.

134. Mori, A., et al., *Avascular necrosis in the femoral head secondary to bone marrow infarction in a patient with graft-versus-host disease after unrelated bone marrow transplantation.* Ann Hematol, 2001. **80**(4): p. 238-42.

135. Torii, Y., et al., *Osteonecrosis of the femoral head after allogeneic bone marrow transplantation.* Clin Orthop Relat Res, 2001(382): p. 124-32.

136. Shimoni, A., et al., *Secondary malignancies after allogeneic stem-cell transplantation in the era of reduced-intensity conditioning; the incidence is not reduced.* Leukemia, 2012.

137. Afessa, B. and S.G. Peters, *Major complications following hematopoietic stem cell transplantation.* Semin Respir Crit Care Med, 2006. **27**(3): p. 297-309.

138. Soubani, A.O., *Critical care considerations of hematopoietic stem cell transplantation.* Crit Care Med, 2006. **34**(9 Suppl): p. S251-67.

139. Huynh, T.N., et al., *Outcome and prognostic indicators of patients with hematopoietic stem cell transplants admitted to the intensive care unit.* J Transplant, 2009. **2009**: p. 917294.

140. Hassan, Z., et al., *Hemorrhagic cystitis: a retrospective single-center survey.* Clin Transplant, 2007. **21**(5): p. 659-67.

141. Garming-Legert, K., et al., *Long-term salivary function after conditioning with busulfan, fractionated or single-dose TBI.* Oral Dis, 2011. **17**(7): p. 670-6.

142. Brown, R.A., et al., *Long-term follow-up of high-risk allogeneic peripheral-blood stem-cell transplant recipients: graft-versus-host disease and transplant-related mortality.* J Clin Oncol, 1999. **17**(3): p. 806-12.

143. Ferrara, J.L. and H.J. Deeg, *Graft-versus-host disease*. N Engl J Med, 1991. **324**(10): p. 667-74.

144. Mielcarek, M., et al., *Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation.* Blood, 2003. **102**(2): p. 756-62.

145. Valks, R., et al., *Late appearance of acute graft-vs-host disease after suspending or tapering immunosuppressive drugs.* Arch Dermatol, 2001. **137**(1): p. 61-5.

146. Bensinger, W.I., D. Maloney, and R. Storb, *Allogeneic hematopoietic cell transplantation for multiple myeloma.* Semin Hematol, 2001. **38**(3): p. 243-9.

147. Zeiser, R., et al., *Immunopathogenesis of acute graft-versus-host disease: implications for novel preventive and therapeutic strategies.* Ann Hematol, 2004. **83**(9): p. 551-65.

148. Shlomchik, W.D., *Graft-versus-host disease.* Nat Rev Immunol, 2007. **7**(5): p. 340-52.

149. Bacigalupo, A., *Management of acute graft-versus-host disease.* Br J Haematol, 2007. **137**(2): p. 87-98.

150. Morris, E.S. and G.R. Hill, *Advances in the understanding of acute graft-versus-host disease.* Br J Haematol, 2007. **137**(1): p. 3-19.

151. Powles, R.L., et al., *Cyclosporin A to prevent graft-versus-host disease in man after allogeneic bone-marrow transplantation.* Lancet, 1980. **1**(8164): p. 327-9.

152. Ringden, O., *Cyclosporine in allogeneic bone marrow transplantation.* Transplantation, 1986. **42**(5): p. 445-52.

153. Reddy, P. and J.L. Ferrara, *Immunobiology of acute graft-versus-host disease.* Blood Rev, 2003. **17**(4): p. 187-94.

154. Hill, G.R. and J.L. Ferrara, *The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation.* Blood, 2000. **95**(9): p. 2754-9.

155. Billingham, R.E., *The biology of graft-versus-host reactions.* Harvey Lect, 1966. **62**: p. 21-78.

156. Korngold, R. and J. Sprent, *Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow.* J Exp Med, 1978. **148**(6): p. 1687-98.

157. Kataoka, Y., et al., *The role of donor T cells for target organ injuries in acute and chronic graft-versus-host disease.* Immunology, 2001. **103**(3): p. 310-8.

158. Welniak, L.A., B.R. Blazar, and W.J. Murphy, *Immunobiology of allogeneic hematopoietic stem cell transplantation.* Annu Rev Immunol, 2007. **25**: p. 139-70.

159. Antin, J.H. and J.L. Ferrara, *Cytokine dysregulation and acute graft-versus-host disease.* Blood, 1992. **80**(12): p. 2964-8.

160. Teshima, T., et al., *Acute graft-versus-host disease does not require alloantigen expression on host epithelium.* Nat Med, 2002. **8**(6): p. 575-81.

161. Beilhack, A., et al., *In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets.* Blood, 2005. **106**(3): p. 1113-22.

162. Panoskaltsis-Mortari, A., et al., *In vivo imaging of graft-versus-host-disease in mice.* Blood, 2004. **103**(9): p. 3590-8.

163. Ferrara, J.L., K.R. Cooke, and T. Teshima, *The pathophysiology of acute graft-versus-host disease.* Int J Hematol, 2003. **78**(3): p. 181-7.

164. Ferrara, J.L. and P. Reddy, *Pathophysiology of graft-versus-host disease.* Semin Hematol, 2006. **43**(1): p. 3-10.

165. Travnik, R., et al., *[Graft-versus-Host Disease (GvHD) - an update : Part 1: Pathophysiology, clinical features and classification of GvHD]*. Hautarzt, 2011. **62**(2): p. 139-54; quiz 155.

166. Ferrara, J.L., *Pathogenesis of acute graft-versus-host disease: cytokines and cellular effectors.* J Hematother Stem Cell Res, 2000. **9**(3): p. 299-306.

167. Vogelsang, G.B., L. Lee, and D.M. Bensen-Kennedy, *Pathogenesis and treatment of graft-versus-host disease after bone marrow transplant.* Annu Rev Med, 2003. **54**: p. 29-52.

168. Jacobsohn, D.A. and G.B. Vogelsang, *Acute graft versus host disease.* Orphanet J Rare Dis, 2007. **2**: p. 35.

169. Rappaport, H., et al., *Histopathologic sequence of events in adult mice undergoing lethal graft-versus-host reaction developed across H-2 and/or non-H-2 histocompatibility barriers.* Am J Pathol, 1979. **96**(1): p. 121-42.

170. Hill, G.R., et al., *Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines.* Blood, 1997. **90**(8): p. 3204-13.

171. Ringden, O., et al., *A randomized trial comparing busulfan vs total body irradiation in allogeneic marrow transplant recipients with hematological malignancies.* Transplant Proc, 1994. **26**(3): p. 1831-2.

172. Levey, A.S. and J. Coresh, *Chronic kidney disease.* Lancet, 2012. **379**(9811): p. 165-80.

173. Vassalotti, J.A., L.A. Stevens, and A.S. Levey, *Testing for chronic kidney disease: a position statement from the National Kidney Foundation.* Am J Kidney Dis, 2007. **50**(2): p. 169-80.

174. Stevens, L.A. and A.S. Levey, *Current status and future perspectives for CKD testing.* Am J Kidney Dis, 2009. **53**(3 Suppl 3): p. S17-26.

175. Walser, M., *Creatinine excretion as a measure of protein nutrition in adults of varying age.* JPEN J Parenter Enteral Nutr, 1987. **11**(5 Suppl): p. 73S-78S.

176. Sarnak, M.J. and B.L. Jaber, *Mortality caused by sepsis in patients with end-stage renal disease compared with the general population.* Kidney Int, 2000. **58**(4): p. 1758-64.

177. Kato, S., et al., *Aspects of immune dysfunction in end-stage renal disease.* Clin J Am Soc Nephrol, 2008. **3**(5): p. 1526-33.

178. Cohen, G., M. Haag-Weber, and W.H. Horl, *Immune dysfunction in uremia.* Kidney Int Suppl, 1997. **62**: p. S79-82.

179. Horl, W.H., et al., *Physicochemical characterization of a polypeptide present in uremic serum that inhibits the biological activity of polymorphonuclear cells.* Proc Natl Acad Sci U S A, 1990. **87**(16): p. 6353-7.

180. Jaber, B.L., et al., *Apoptosis of leukocytes: basic concepts and implications in uremia.* Kidney Int Suppl, 2001. **78**: p. S197-205.

181. Jaber, B.L., et al., *Mechanisms of neutrophil apoptosis in uremia and relevance of the Fas (APO-1, CD95)/Fas ligand system.* J Leukoc Biol, 2001. **69**(6): p. 1006-12.

182. Kohler, H., et al., *Active hepatitis B vaccination of dialysis patients and medical staff.* Kidney Int, 1984. **25**(1): p. 124-8.

183. Kreft, B., et al., *Low efficiency of active immunization against diphtheria in chronic hemodialysis patients.* Kidney Int, 1997. **52**(1): p. 212-6.

184. Rautenberg, P., et al., *Influenza subtype-specific immunoglobulin A and G responses after booster versus one double-dose vaccination in hemodialysis patients.* Eur J Clin Microbiol Infect Dis, 1989. **8**(10): p. 897-900.

185. Girndt, M., M. Pietsch, and H. Kohler, *Tetanus immunization and its association to hepatitis B vaccination in patients with chronic renal failure.* Am J Kidney Dis, 1995. **26**(3): p. 454-60.

186. Haag-Weber, M. and W.H. Horl, *Dysfunction of polymorphonuclear leukocytes in uremia.* Semin Nephrol, 1996. **16**(3): p. 192-201.

187. Horl, W.H., *Neutrophil function and infections in uremia.* Am J Kidney Dis, 1999. **33**(2): p. xlv-xlviii.

188. Yilmaz, M.I., et al., *Low-grade inflammation in chronic kidney disease patients before the start of renal replacement therapy: sources and consequences.* Clin Nephrol, 2007. **68**(1): p. 1-9.

189. Meuer, S.C., et al., *Selective blockade of the antigen-receptor-mediated pathway of T cell activation in patients with impaired primary immune responses.* J Clin Invest, 1987. **80**(3): p. 743-9.

190. Girndt, M., et al., *T cell activation defect in hemodialysis patients: evidence for a role of the B7/CD28 pathway.* Kidney Int, 1993. **44**(2): p. 359-65.

191. Stenvinkel, P., et al., *IL-10, IL-6, and TNF-alpha: central factors in the altered cytokine network of uremia--the good, the bad, and the ugly.* Kidney Int, 2005. **67**(4): p. 1216-33.

192. Kimmel, P.L., et al., *Immunologic function and survival in hemodialysis patients.* Kidney Int, 1998. **54**(1): p. 236-44.

193. Schiffrin, E.L., M.L. Lipman, and J.F. Mann, *Chronic kidney disease: effects on the cardiovascular system.* Circulation, 2007. **116**(1): p. 85-97.

194. Go, A.S., et al., *Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization.* N Engl J Med, 2004. **351**(13): p. 1296-305.

195. Amann, K., et al., *Special characteristics of atherosclerosis in chronic renal failure.* Clin Nephrol, 2003. **60 Suppl 1**: p. S13-21.

196. Imamura, M., et al., *Serum cytokine levels in bone marrow transplantation: synergistic interaction of interleukin-6, interferon-gamma, and tumor necrosis factor-alpha in graft-versus-host disease.* Bone Marrow Transplant, 1994. **13**(6): p. 745-51.

197. Wiendl, H., et al., *Human muscle cells express a B7-related molecule, B7-H1, with strong negative immune regulatory potential: a novel mechanism of counterbalancing the immune attack in idiopathic inflammatory myopathies.* FASEB J, 2003. **17**(13): p. 1892-4.

198. Buchali, A., et al., *Immediate toxicity during fractionated total body irradiation as conditioning for bone marrow transplantation.* Radiother Oncol, 2000. **54**(2): p. 157-62.

199. Soubani, A.O., K.B. Miller, and P.M. Hassoun, *Pulmonary complications of bone marrow transplantation.* Chest, 1996. **109**(4): p. 1066-77.

200. Siegfried, E., T.B. Chou, and N. Perrimon, *wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate.* Cell, 1992. **71**(7): p. 1167-79.

201. Wang, Y., et al., *A large family of putative transmembrane receptors homologous to the product of the Drosophila tissue polarity gene frizzled.* J Biol Chem, 1996. **271**(8): p. 4468-76.

202. Wodarz, A. and R. Nusse, *Mechanisms of Wnt signaling in development.* Annu Rev Cell Dev Biol, 1998. **14**: p. 59-88.

203. Huelsken, J. and J. Behrens, *The Wnt signalling pathway.* J Cell Sci, 2002. **115**(Pt 21): p. 3977-8.

204. Behrens, J., et al., *Functional interaction of beta-catenin with the transcription factor LEF-1.* Nature, 1996. **382**(6592): p. 638-42.

205. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annu Rev Cell Dev Biol, 2004. **20**: p. 781-810.

206. Gordon, M.D. and R. Nusse, *Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors.* J Biol Chem, 2006. **281**(32): p. 22429-33.

207. Schena, M., et al., *Quantitative monitoring of gene expression patterns with a complementary DNA microarray.* Science, 1995. **270**(5235): p. 467-70.

208. Slonim, D.K. and I. Yanai, *Getting started in gene expression microarray analysis.* PLoS Comput Biol, 2009. **5**(10): p. e1000543.

209. Holler, E., et al., *Modulation of acute graft-versus-host-disease after allogeneic bone marrow transplantation by tumor necrosis factor alpha (TNF alpha) release in the course of pretransplant conditioning: role of conditioning regimens and prophylactic application of a monoclonal antibody neutralizing human TNF alpha (MAK 195F).* Blood, 1995. **86**(3): p. 890-9.

210. Teshima, T. and J.L. Ferrara, *Understanding the alloresponse: new approaches to graft-versus-host disease prevention.* Semin Hematol, 2002. **39**(1): p. 15-22.

211. Mapara, M.Y., et al., *Expression of chemokines in GVHD target organs is influenced by conditioning and genetic factors and amplified by GVHR.* Biol Blood Marrow Transplant, 2006. **12**(6): p. 623-34.

212. Sadeghi, B., et al., *Expansion and activation kinetics of immune cells during early phase of GVHD in mouse model based on chemotherapy conditioning.* Clin Dev Immunol, 2010. **2010**: p. 142943.

213. Asakura, S., et al., *Alloantigen expression on non-hematopoietic cells reduces graft-versus-leukemia effects in mice.* J Clin Invest, 2010. **120**(7): p. 2370-8.

214. Schilbach, K., et al., *PD-1 - PD-L1 pathway is involved in suppressing alloreactivity of heart infiltrating T cells during murine GVHD across minor histocompatibility antigen barriers.* Transplantation, 2007. **84**(2): p. 214-222.

215. Eyrich, M., et al., *Sequential expression of adhesion and costimulatory molecules in graft-versus-host disease target organs after murine bone marrow transplantation across minor histocompatibility antigen barriers.* Biol Blood Marrow Transplant, 2005. **11**(5): p. 371-82.

216. Keir, M.E., et al., *Tissue expression of PD-L1 mediates peripheral T cell tolerance.* J Exp Med, 2006. **203**(4): p. 883-95.

217. Grabie, N., et al., *Endothelial programmed death-1 ligand 1 (PD-L1) regulates CD8+ T-cell mediated injury in the heart.* Circulation, 2007. **116**(18): p. 2062-71.

218. Dong, H. and X. Chen, *Immunoregulatory role of B7-H1 in chronicity of inflammatory responses.* Cell Mol Immunol, 2006. **3**(3): p. 179-87.

219. Stevens, A.M., K.M. Sullivan, and J.L. Nelson, *Polymyositis as a manifestation of chronic graft-versus-host disease.* Rheumatology, 2003. **42**(1): p. 34-39.

220. Marsh, L.M., et al., *Surface expression of CD74 by type II alveolar epithelial cells: a potential mechanism for macrophage migration inhibitory factor-induced epithelial repair.* Am J Physiol Lung Cell Mol Physiol, 2009. **296**(3): p. L442-52.

221. Balla, S., M.B. Nusair, and M.A. Alpert, *Risk factors for atherosclerosis in patients with chronic kidney disease:recognition and management.* Curr Opin Pharmacol, 2013.

222. Drueke, T.B. and Z.A. Massy, *Atherosclerosis in CKD: differences from the general population.* Nat Rev Nephrol, 2010. **6**(12): p. 723-35.

223. Shoji, T., et al., *Chronic kidney disease, dyslipidemia, and atherosclerosis.* J Atheroscler Thromb, 2012. **19**(4): p. 299-315.

224. Osterud, B. and E. Bjorklid, *Role of monocytes in atherogenesis.* Physiol Rev, 2003. **83**(4): p. 1069-112.

225. Lee, D.K., et al., *Activation of the canonical Wnt/beta-catenin pathway enhances monocyte adhesion to endothelial cells.* Biochem Biophys Res Commun, 2006. **347**(1): p. 109-16.

226. Tickenbrock, L., et al., *Wnt signaling regulates transendothelial migration of monocytes.* J Leukoc Biol, 2006. **79**(6): p. 1306-13.

227. Pereira, C., et al., *Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the antiinflammatory action of activated protein C and interleukin-10.* Arterioscler Thromb Vasc Biol, 2008. **28**(3): p. 504-10.